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"Potential inhibitors for a mutation in the KCNJ6 gene."

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Abstract

GIRK2, which is also called Kir3.2 is a G-protein-activated inward rectifier potassium channel encoded by the KCNJ6 gene. GIRK2 channels generate small, hyperpolarising currents that inhibit neuronal activity. (Horvath et al., 2018) They limit the outward flow of K^+ ions by blocking the pore by intracellular Mg^{2+} and polyamines. (Hibino et al., 2010)
Kir channels in general are essential for maintaining the resting membrane potential and for regulating the excitability of the cells. (Hibino et al., 2010)

Mutations in the KCNJ6 gene (encoding GIRK2 channels) are linked to Keppen-Lubinsky syndrome or related diseases. (Masotti et al., 2015; Horvath et al., 2018)
Gain-of-function mutations cause abnormal inward currents that are no longer G-protein-coupled, lose K^+ selectivity and gain permeability for other cations, in particular Na^+. These altered channel characteristics cause sustained depolarisation, instead of the generally inhibitory response of GIRK channels. (Horvath et al., 2018) Currently, no therapies for these patients exist.
Thus, inhibitors for these mutated GIRK2 channels could be interesting and promising drug candidates for the treatment of neurological disorders that are connected to neuronal hyperexcitability, such as the Keppen-Lubinsky syndrome.

A recent abstract, published in 2020, determined that the following six compounds: XE991, M084, M204, ML297, RTG and RL648-81, block GIRK2, however the binding sites and mode of these compounds is unknown. (De la Rosa and Shapiro, 2020)
This diploma study aims to find appropriate binding sites for each of the compounds and rank them according to their binding affinity. Docking investigations revealed that except for one compound all are predicted to bind to the central transmembrane cavity, with ML297 and XE991 having highest affinity.

An important aspect for future studies is to perform experimental validation, to find out if the compounds actually bind the disease mutants as well.

Zusammenfassung

GIRK2, auch Kir3.2 genannt, ist ein G-Protein-aktivierter einwärts gleichrichtender Kaliumkanal, der durch das KCNJ6 Gen codiert ist. GIRK2 Kanäle erzeugen kleine, hyperpolarisierenden Kaliumauswärtsstrom, der die neuronale Aktivität inhibiert. (Horvath et al., 2018) Sie verhindern den Auswärtsstrom von Kaliumionen, indem sie die Pore mit intrazellulärem Mg^{2+} und Polyaminen blockieren. (Hibino et al., 2010)
Im Allgemeinen sind Kir Kanäle daran beteiligt das Ruhemembranpotential zu erhalten und die Erregbarkeit von Zellen zu regulieren.

Mutationen im KCNJ6 Gen, die den GIRK2 Kanal codieren, sind mit dem Keppen-Lubinsky-Syndrom oder verwandten Krankheiten verbunden. (Masotti et al., 2015; Horvath et al., 2018) Gain-of-Function-Mutationen führen zu abnormalen Einwärtsströmen, die nicht mehr G-Protein-gekoppelt sind, ihre K^+-Selektivität verlieren und Permeabilität für andere Kationen, insbesondere Na^+, gewinnen. Diese veränderten Kanaleigenschaften verursachten anhaltende Depolarisation, statt der für gewöhnlich inhibierenden Antwort der GIRK Kanäle. (Horvath et al., 2018) Derzeit gibt es keine Therapien für diese Patienten.
Deswegen wären Inhibitoren für diesen mutierten GIRK2 Kanal interessante und vielversprechende Wirkstoffe für die Behandlung von neurologischen Erkrankungen, die mit neurologischer Übererregbarkeit verbunden sind, sowie zum Beispiel das Keppen-Lubinsky Syndrom.

In einem kürzlich veröffentlichten Abstract aus dem Jahr 2020 wurde festgestellt, dass die folgenden sechs Verbindungen: XE991, M084, M204, ML297, RTG und RL648-81, GIRK2 blockieren, wobei jedoch die Bindungsstellen und der Modus dieser Verbindungen unbekannt sind. (De la Rosa and Shapiro, 2020)
Diese Diplomarbeit hat das Ziel geeignete Bindungsstellen für jedes der Compounds zu finden und sie nach ihrer Bindungsaffinität zu bewerten und zu reihen. Docking Untersuchungen ergaben, dass mit Ausnahme einer Verbindung alle anderen an den zentralen transmembranären Hohlraum binden werden, wobei ML297 und XE991 die höchste Affinität aufweisen.

Ein wichtiger Aspekt für zukünftige Studien ist die experimentelle Validierung, um herauszufinden, ob die Verbindungen tatsächlich auch die Krankheitsmutanten binden.

Acknowledgement

First and foremost, I would like to express my gratitude to my supervisor, Prof. Mag. Dr. Anna Weinzinger for always taking the time to help me work on this interesting topic. Despite of the current COVID-19 situation, she was there for me throughout my whole diploma thesis, whenever I had questions about my research or about the computational methods I used.

I want to thank my parents, Gaklin and Lotfy, for always supporting and encouraging me throughout my years of study. This achievement would not have been possible without them. Thank you!
Also, I want to thank my brothers, Thomas and Minas, my family in Egypt and all my dear friends for believing in me and making all these years of study more enjoyable.

Lastly, I want to thank God, because I felt His great support in all these years and my faith in Him always gave me strength.

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1. Introduction

1.1. Ion channels

Ion channels are membrane proteins that create aqueous pores allowing ions of appropriate size and charge to pass the lipid bilayer along their electrochemical gradients. (Alberts et al., 2002) They have various functions such as maintaining the resting membrane potential, inducing action potential and more electrical signals by regulating the flow of ions. (Abdul Kadir et al., 2018; Alexander SP et al., 2011)
Ion channels are expressed in many, if not all living cells, to pass charged ions through the lipid cell membrane, which is otherwise not permeable for them. (Huettner et al., 2013)
In many physiological processes, ion channels play an essential role. For instance, in the contraction of skeletal muscles and of the heart, as well as in the nervous system. (Huettner et al., 2013)

In comparison to other forms of ion transporters, ion channels have two typical features. They transport much more ions through the channel (more than 106 ions per second), and the ions pass through these channels along their electrochemical gradient passively without using metabolic energy (such as co-transport, active transport, or ATP). (Hille B, 2001)

Ion channels are selective transporters that only allow ions of specific charge or size to permeate through. (Purves D et al., 2001; Hille B and Catterall WA, 1999) Ions in solution need to be stabilised by polarised water molecules around them. Highly selective ion channels line the narrow inside of the pore with polarised carbonyl oxygen atoms, creating a hydrophilic environment. If the pores of the channel are wide enough to pass water and ions through at the same time, resulting in less selective channels. (Huettner et al., 2013)

Ion channels open either in response to a change in membrane potential (voltage-gated) or due to the binding of a particular ligand (transmitter-gated). (Alberts et al., 2002)
Voltage-gated cation channels in electrically excitable cells generate self-amplifying action potentials. Transmitter-gated ion channels transform chemical binding of a ligand to electrical signals. For example, glutamate and acetylcholine open transmitter-gated cation channels, leading to depolarisation of the postsynaptic membrane, making them excitatory neurotransmitters. Other neurotransmitters such as GABA and Glycine, on the other hand,

open Cl^- or K^+ channels, which polarise the postsynaptic membrane inhibiting action potential. (Alberts et al., 2002)

Many various types of ion channels, with different gating mechanisms and different selectivity, are expressed in cells of higher organisms. (Huettner et al., 2013)
They can be categorised by different characteristics, like their mechanism of opening and closing, the species of ions they permeate, how many pores they have and where they are expressed. (Alberts et al., 2002)
A general way to classify them is by gating: a change in membrane potential gates voltage-gated channels. Ligand-gated ion channels react to the chemical bonding of a neurotransmitter with a change in conformation of their structure, leading them to either open or close. These channels are also referred to as ionotropic channels. Second messengers are also possible ligands in this category. Lipid-gated ion channels respond to the binding of specific lipid molecules, such as the well-characterised PIP2 (Phosphatidylinositol-4,5-bisphosphate) or others. A lot of K^+ channels belong to this group. (Hansen et al., 2011; Hansen, 2015)
Mechanically gated ion channels are linked to the cytoskeleton through a cytoplasmic extension. (Alberts et al., 2002)

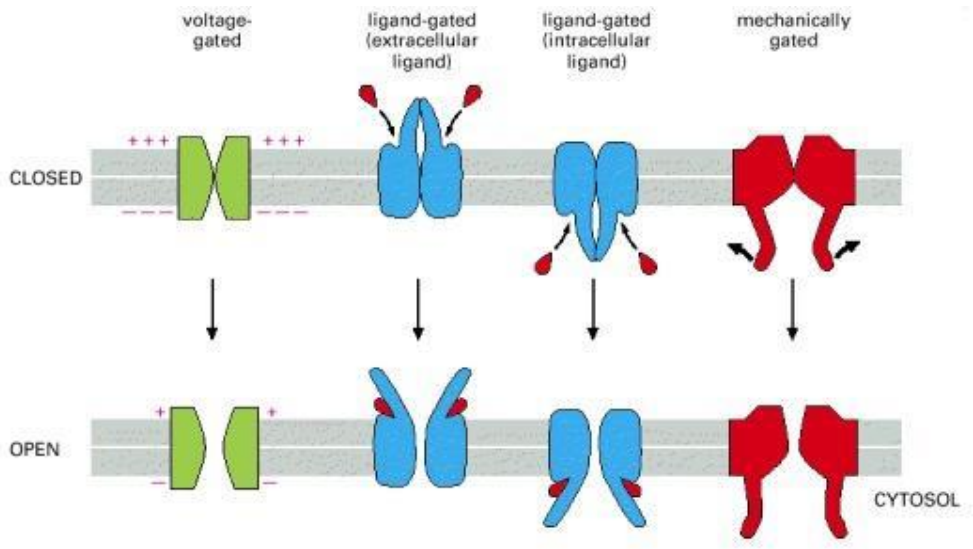


Figure 1: Ion channel- gating (Alberts et al., 2002)

The nerve cell (neurons)

Neurons are the cells that make the most use of ion channels. (Alberts et al., 2002) The primary function of a neuron is receiving, conducting and transmitting various signals. To be able to carry out this task nerve cells are very elongated. All neurons have a cell body with a nucleus and several short dendrites, one long axon, which conducts information away from the cell body to different targets. Axons transport signals away from the cell body, while dendrites receive data from an axon of other nerve cells. Branches of the axon of a nerve cell can end on neurons or different types of cells, for example, muscle or gland cells. (Alberts et al., 2002)

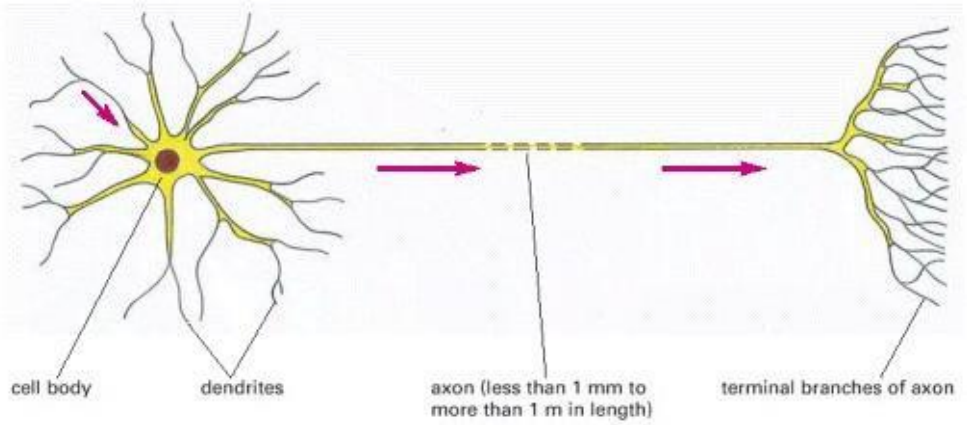


Figure 2: A vertebrate nerve cell (Alberts et al., 2002)

The chemical synapse

Neurons communicate at specialised points of contact called synapses. They connect indirectly and are electrically isolated to each other, separated by a narrow synaptic cleft. When the membrane potential of the presynaptic cell changes, neurotransmitters are released, which are located in vesicles inside the cell and are emitted by exocytosis. Neurotransmitters then bind to transmitter-gated ion channels in the postsynaptic cell and change their electrical potential. Immediately after their release neurotransmitters are removed from the synaptic cleft either by certain enzymes or by their reuptake through the presynaptic cell. This removal of neurotransmitters makes sure that neighbour cells do not get influenced by them and also to clear the synaptic cleft before the next pulse is emitted, ensuring accurate communication between the cells. (Alberts et al., 2002)

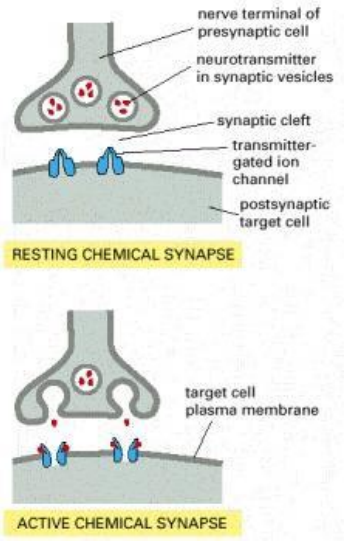


Figure 3: A chemical synapse (Alberts et al., 2002)

The response changes, depending on which type of transmitter-gated ion channels are located on the postsynaptic cell.

Excitatory neurotransmitters cause cation channels to open, letting Na^+ in and depolarising the postsynaptic membrane, firing an action potential. Inhibitory neurotransmitters, on the other hand, open K^+ or Cl^- channels suppressing excitatory neurotransmitters to cause depolarisation. (Alberts et al., 2002)

Therapeutic relevance

In many pathological conditions, ion channels are directly or indirectly changed, leading to channelopathies. Therefore, they are common drug targets for a variety of diseases. Experimental studies of interactions between the channel and drugs have shown that mutations in the ion channel can decrease or increase affinity for the drug, probably changing its therapeutic action. (Camerino et al., 2007)

The more ion channels are investigated, and channelopathies are identified, the number of possible treatments for neurological channelopathies will grow. (Camerino et al., 2007)

Patch-clamp is the gold standard technology to investigate the pharmacology of ion channels. Recordings of this technique have excellent signal and time resolutions, making it possible to get useful information about the drug-channel interaction. The only drawback is the laborious procedure, that needs to be done, making this technology not suitable to be used as a screening platform. (Camerino et al., 2007)

There are already many therapeutic drugs on the market, targeting ion channels either directly or indirectly. Examples are local anaesthetics, sulfonylurea derivatives and benzodiazepines. (Huettner et al., 2013)

1.2. Potassium channels

Probably the most common of all ion channels are those selective for K^+. They can be found in almost all cell membranes and have the vital role of maintaining or setting the resting potential along the membranes. (Alberts et al., 2002)

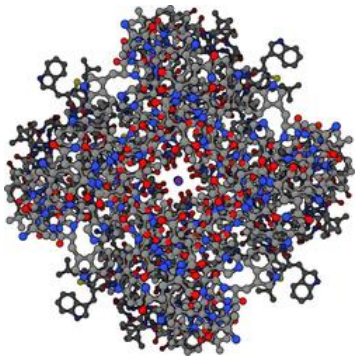


Figure 4: Top view of a K^+ ion channel (Wikipedia: Potassium Channel/ PDB: 1BL8)

Potassium channels are formed out of four subunits arranged around an ion-conducting pore, forming a protein complex. These subunits can be identical and form a homotetrameric, symmetrical complex, or they are not identical, and they form heterotetrameric complexes. (Doyle et al., 1998) Nonetheless, the subunits need to be of the same subfamily. (Horvath et al., 2018)

Potassium channels can be classified into three families:
Voltage-gated K^+ channels help with the repolarisation of the action potential. Defects in these channels are known to cause epilepsy, and a loss of these channels cause a tremendous increase of excitability in neurons.
Two-pore K^+ channels are involved in pain sensation and are also associated with familial migraine with aura.
Inwardly rectifying K^+ channels play an essential role in maintaining the resting membrane potential and in adjusting cell excitability of neuronal and cardiac cells. These ion channels keep the outward flow of K^+ to a voltage range close to the resting potential. (Horvath et al., 2018)

Selectivity filter

When K^+ ions enter the selectivity filter of the channel, their hydration shell is removed. The highly conserved "signature sequence" in K^+ channels is five residues long (TXGYG), and it is present in all of the four subunits. (Hibino et al., 2010)

Towards the middle of the pore, electronegative carbonyl oxygen atoms get aligned and create a square anti-prism that mimics the water-solvating shell around K^+ ions. The space between the K^+ ions and the carbonyl oxygens in the pore is equal to the space between K^+ ions in solution and water oxygen in the hydration shell. This makes the route through the pore of potassium channels energetically favourable for K^+ ions. Na^+ ions are not big enough to fill the distance to the carbonyl oxygen atoms, making it more profitable for them to stay in the extracellular water solution. (Lodish H et al., 2016)

Structure of a bacterial potassium channel

It has long been unknown how these ion channels are highly selective as well as high in conductance. Even if just one single amino acid is changed in the sequence of the pore, it can lead to a loss in ion selectivity or cell death. Pore size cannot be the reason why K^+ channels are selective for K^+ ions because Na^+ ions are smaller in size. It was also not clear how the conductance rate can be high, because the binding sites for K^+ have a high affinity for these ions and should slow their passage.

When the bacterial K^+ channel was structurally elucidated by X-ray crystallography, these uncertainties were solved. Four transmembrane subunits form the pore in the middle of the ion channel. (Alberts et al., 2002)

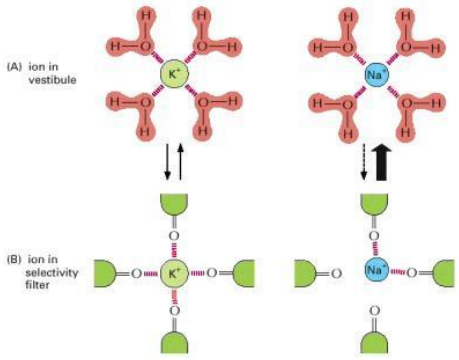


Figure 5: Selectivity filter in potassium channels (Alberts et al., 2002)

In the cytosolic part, where ions enter the pore, mainly negatively charged amino acids are located to attract cations. There are two transmembrane helices in a subunit, slightly tilted to the outside, creating a cone with the broader end at the exit of the ions. A polypeptide chain connects both of the transmembrane helices in a subunit that forms an ⟨ helix and another

essential loop that makes up the selectivity filter. The amino acid sequence of the selectivity loops is highly conserved in all potassium channels. (Alberts et al., 2002)

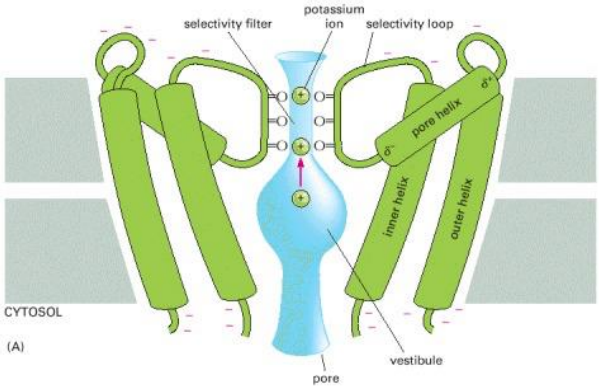


Figure 6: Two of the four subunits (Alberts et al., 2002)

In the crystal structure of this bacterial K^+ channel, two K^+ ions are going through the selectivity filter; the second one 8Å behind. Repulsion between the K^+ ions is very likely the reason for them to move forward through the pore. (Alberts et al., 2002)

Selectivity filter loops are not flexible, and their conformation does not change by opening or closing of the channel. On the other hand, transmembrane helices forming the other parts of the pore do change their conformation with the closure of the ion channel. The pore remains slightly open, even in the closed state, but ions cannot enter, because of hydrophobic amino acid side chains lining the inside of the pore. (Alberts et al., 2002)

Blockers

K^+ channel blockers inhibit K^+ ions from passing through the channel pore. They can bind to the selectivity filter and prevent K^+ ions from binding, or they can hinder ions from passing by binding to the outside of the filter. Quaternary ammonium, for example, is a competitor, which binds to the central cavity of the ion channel, blocking already opened K^+ channels. Barium ions bind to the selectivity filter with high affinity inhibiting K^+ ions from getting through the channel. (Luzhkov et al., 2005; Piasta et al., 2011)

1.3. Kir channel

Inwardly rectifying potassium (IRK) channels have the function of maintaining the resting membrane potential and regulating cell excitability. (Hibino et al., 2010; Lüscher and Slesinger, 2010) They are not sensitive to changes in membrane potential, unlike the voltage-gated K^+ channels and they keep the K^+ outward flow to a range close to the resting potential. (Hibino et al., 2010; Lüscher and Slesinger, 2010)

Kir channels are involved in many physiological functions, such as neuronal signalling, insulin secretion, kidney function and heart rate regulation. (Hibino et al., 2010; Choi et al., 2011) Inwardly rectifying potassium channels are gated by specific lipids; for example, PIP2 (Phosphatidylinositol-4,5-bisphosphate) can activate them. Some diseases have been connected to the dysfunction of these channels. (Hansen, 2015; Abraham et al., 1999)

Seven Kir families are known (Kir1.x-Kir7.x). Kir channels assemble to homotetramers or heterotetramers, only using members of the same subfamily. For instance, Kir3.2 cannot form a tetramer with Kir2.1, but it can assemble with Kir3.1. (Hibino et al., 2010; Lüscher and Slesinger, 2010) Kir channels can either have high basal activity, meaning that they are constitutively open), for example, Kir2, or they may have low basal activity, Kir3, needing ligands, such as G-proteins or alcohol to be enhanced. (Hibino et al., 2010; Lüscher and Slesinger, 2010)

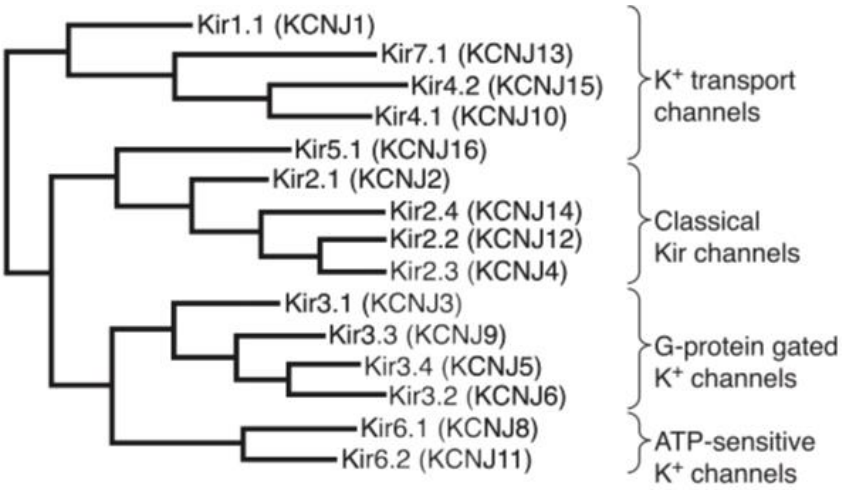


Figure 7: Phylogenetic tree of Kir channels (Hibino et al., 2010)

Until now, 15 human Kir subunit genes have been found and categorised into four functional groups: K^+ transport, classical, G-protein-gated (Kir3.x) and ATP-sensitive K^+ channels. (Hibino et al., 2010)

Molecular structure

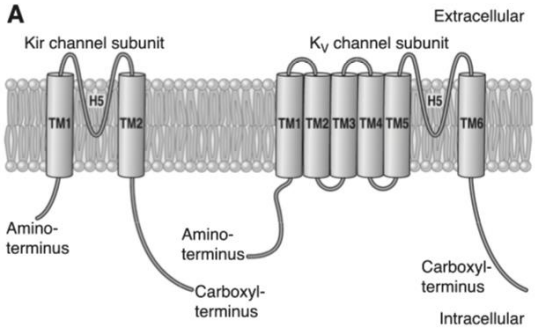


Figure 8: Primary structure of a Kir channel subunit (Hibino et al., 2010)

By expression-cloning technologies, two Kir channel cDNAs could be isolated in 1993 (Kir1.1 and Kir2.1). Both showed two transmembrane segments linked by a pore-forming region (H5), as well as a cytoplasmic amino and a carboxy terminus. (Fig. 8) The primary structure, as described above, is known as the "basic building block" and it is found in all Kir channel types. The pore-forming region (H5) functions as the ion selectivity filter and has a highly conserved "signature sequence" in all K^+-selective channels (TXGYG). The S4 voltage-sensitive region that is conserved in voltage-gated ion channels is non-existent in Kir channels. This means that these channels lack voltage-sensitivity, making them able to limit the outward flow of K^+ ions to a range close to the resting membrane potential. (Hibino et al., 2010)

Inward rectification of Kir channels is their defining quality. But this is not an intrinsic function of the channel, but it is rather due to the block of the outward current by intracellular molecules. (Hibino et al., 2010) In general, Kir channels have a higher conductance of K^+ ions at negative membrane potentials. When the membrane potential reaches positive values, an inhibition of the outward flow, caused by Mg^{2+} and polyamines, occurs. (Lopatin et al., 1995; Villa and Combi, 2016)

1.3.1. G-protein-gated K^+ channels

G-protein-gated K^+ (GIRK) channels, also referred to as Kir3.x channels, belong to the inwardly rectifying K^+ channels, which limit the outward flow of K^+ ions by blocking the pore by intracellular Mg^{2+} and polyamines. (Hibino et al., 2010)

For all Kir channels, the anionic lipid PIP2 is necessary for channel activation. (Hibino et al., 2010) They also need G-proteins, together with PIP2, for activation and some channel types further require intracellular Na^+ ions. (Whorton et al., 2011)

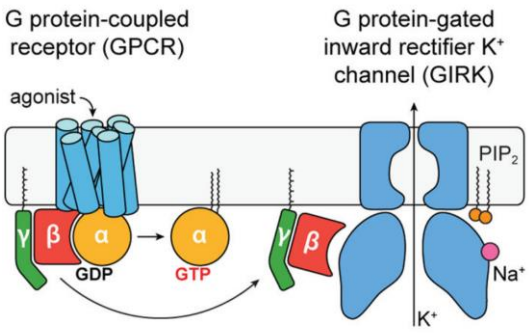


Figure 9: A figure of G-protein-coupled receptor activation of GIRK channels. When agonists bind to the receptor a GDP, bound to a G-protein, is exchanged for GTP causing the G-protein to leave the receptor. Then the Gα and Gβγ subunits dissociate from each other and interact with effector proteins. Gβγ binds to the cytoplasmic part of a GIRK channel when PIP2 is bound, leading the channel to open. GIRK channels can also be activated by high levels of intracellular Na^+ ions. (Whorton et al., 2013)

1.3.2. GIRK2

GIRK2, which is also called Kir3.2, is a G-protein-activated inward rectifier potassium channel which is encoded by the KCNJ6 gene.

GIRK2 generate small, hyperpolarising outward potassium currents that inhibit neuronal activity. (Horvath et al., 2018) They work in response to neurotransmitters, like dopamine, acetylcholine, serotonin, somatostatin, adenosine, opioids and GABA (γ-aminobutyric acid), which stimulate their G-protein-coupled receptors (GPCRs). (Villa et al., 2010)

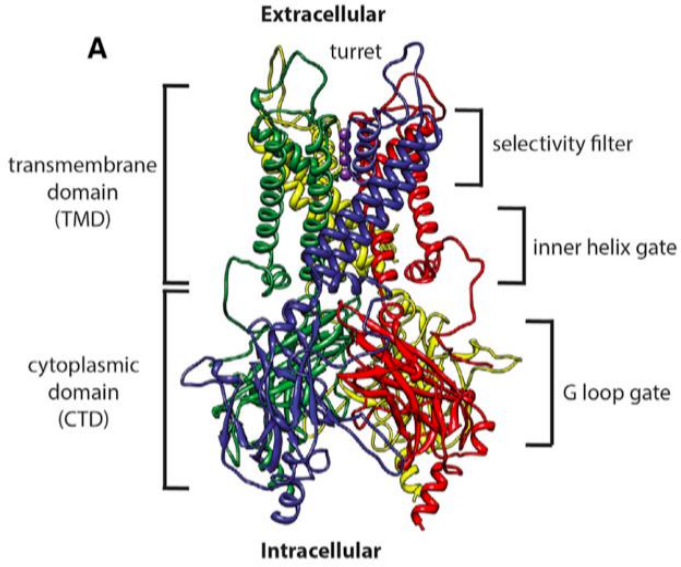


Figure 10: Crystal structure of KCNJ6 (GIRK2), each subunit in a different colour

PDB: 3SYO (Masotti et al., 2015)

A mutation in GIRK2 is known to cause the well-studied weaver mouse mutation. Mice having this mutation are ataxic. They show a degeneration of their dopaminergic neurons, caused by neuroinflammation. Other symptoms are problems in motor coordination and changes in the regional metabolism of the brain. (Peng et al., 2006; Strazielle et al., 2006)

Another less-studied, orphan disease that is caused by a mutation in GIRK2 is the Keppen-Lubinsky syndrome, which will be a main subject of this diploma study and further discussed in the next chapter.

Gating mechanism of GIRK2

To function GIRK channels need to form homo- or heterotetramers. They require PIP_2 to be present so that together with intracellular regulators ($G\beta\gamma$, Na^+) they can maintain and stabilise the open state.

GIRK activation inhibits excitability, slowing the rate of the pacemaker and atrial cell firing in the heart. They inhibit transmitter release by presynaptic neurons and excitation of postsynaptic neurons.

GIRK can be inhibited by hydrolysis or dephosphorylation of PIP_2 due to channel desensitisation that lasts as long as the resynthesis of PIP_2. (Li et al., 2019)

There are three constrictions along the permeation pathway. The selectivity filter, which chooses K^+ over other ions. The inner helix gate, which is also referred to as the helix bundle crossing (HBC) gate and the G-loop gate, a cytosolic gate, unique to Kir channels. (Li et al., 2019)

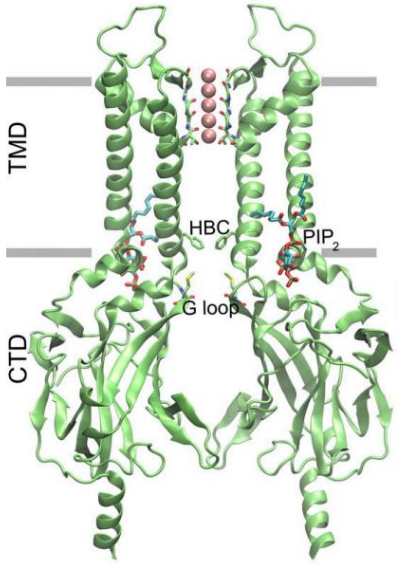


Figure 11: Two opposing subunits of the GIRK2 structure (PDB: 3SYA). Estimated membrane region between TMD and CTD is shown as grey bars. (Bernstein et al., 2019)

PIP_2 stabilises the open state of the ion channel through direct interaction, but regulators are needed to increase affinity to PIP_2, which is unable to gate on its own. Na^+ ions stabilise the G-loop gate in the open state through an anti-clockwise rotation that is able to shift the G-loop gate over the minimum distance required. HBC gate distances are not affected, causing it to be the limiting gate. G$\beta\gamma$ stabilises the HBC gate, and the rocking movement enables a right shift of the HBC gate and the G-loop gate to the open state. Na^+ and G$\beta\gamma$ work together to activate the channel the most, having the greatest P_0 (= open probability). (Li et al., 2019)

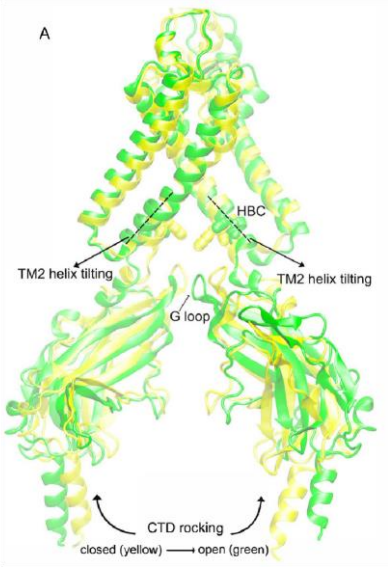


Figure 12: (Li et al., 2019)

There are two important movements controlling the HBC gating, the rocking movement of the C-terminal domain (CTD) and the tilting movement of the transmembrane domain 2 (TM2). The G$\beta\gamma$ dimer helps CTD to rock more compared to the binding of a single Na^+ ion Na^+ has a more distant binding location and is unable to induce strong interactions between the loops. When G$\beta\gamma$ is present, both loops act like a crank. G$\beta\gamma$ binds between the βD2-βE1 loop of one subunit and the βL-βM loop of its adjacent subunit, changing the conformation, causing a local twist. The stronger the twist rotations are, the more the CTD will rock. (Li et al., 2019)

The G-loop gate is stabilised by interactions between the CD-loop (glutamic acid 315) and the β strand (arginine 324). During channel activation by Na^+ this interaction causes glutamic acid-315 to be pulled outwards, causing an anti-clockwise rotation, which is an opening movement. (Li et al., 2019)
Movements of HBC and G-loop gates are sequential but not simultaneous. When the gate opens, the G-loop gate opening precedes. When it closes, HBC closing happens first.
HBC and G-loop gates move in opposite directions. (Li et al., 2019)

2. Mutations in inwardly rectifying potassium channels

In various cells, inwardly rectifying potassium (Kir) channels are important for maintaining the resting membrane potential and for regulating the excitability of the cells. (Hibino et al., 2010)
Genetic neurological channelopathies are a dominantly inherited, heterogeneous group of disorders, changing the function of the channel. (Spillane et al., 2016; Horvath et al., 2018)
Ion channelopathies are involved in various medical conditions, such as movement disorder, paroxysmal dyskinesia, skeletal muscle disorder, periodic paralysis, migraine and epilepsy. (Horvath et al., 2018)

Voltage-gated K^+ channelopathies usually lead to some form of epilepsy and loss of these ion channels to a drastic increase in excitability of neurons. (Villa and Combi, 2016; Horvath et al., 2018) Defects in two-pore K^+ channels are linked to familial migraine with aura and known to contribute to pain sensation. (Horvath et al., 2018)
Inwardly rectifying K^+ channels, such as Kir2.1, Kir3, Kir4 and Kir6, are known to be related to epileptic phenotypes. (Villa and Combi, 2016)

2.1. Mutations in GIRK channels

G-protein-gated Kir channels (Kir3) regulate electrical excitability in neurons and cardiomyocytes. (Slesinger et al., 1995, Villa et al., 2010)
Changes in GIRK channel functions are known to result in severe brain disorders, such as epilepsy. Epilepsy is a chronic neurological disease, affecting 1 to 2% of the population. It is known to cause periodic seizures because of aberrant electrical activity in the CNS (central nervous system). (Steinlein et al., 2004; Villa and Combi, 2016)

To prove that GIRK channels play an essential role in epilepsy, a GIRK2 knockout mouse model was generated and characterised. Compared to the wild type, these knockout mice were more prone to develop seizures. (Signorini et al., 1997; Villa and Combi, 2016)
Another proof for GIRK playing a role in epilepsy was found, when ML-297, a potent activator for GIRK, was administered to mice, causing epileptic attacks to occur. (Kaufmann et al., 2013; Villa and Combi, 2016) Inhibitors of GIRK channels showed similar activity, also causing seizures. (Mazarati et al., 2006; Villa and Combi, 2016)

2.2. Keppen-Lubinsky syndrome

Keppen-Lubinsky syndrome (KPLBS) is an orphan disease, first described in 2001. Symptoms of this rare disorder are severe developmental delay and intellectual disability, overactivity of physiological reflexes, hypertonia, abnormally small head, large bulging eyes, a thin nasal bridge, a tented upper lip, an opened mouth, a high palate, tightly adherent skin, and an aged appearance. Another hallmark of KPLBS is lipodystrophy, which can either be generalised or restricted to facial adipose tissue. (Masotti et al., 2015)

The exomes of the three unrelated patients with the Keppen-Lubinsky syndrome were sequenced, and de novo mutations in the KCNJ6 (GIRK2) gene was found.
In two individuals, the amino acid Threonine 152 was completely deleted (p.Thr152del), and one patient had a Glycine changed to a Serine (p.Gly154Ser). These mutations are known to impair the proper functioning of this GIRK2 channel. (Masotti et al., 2015)

2.2.1. p.Thr152del

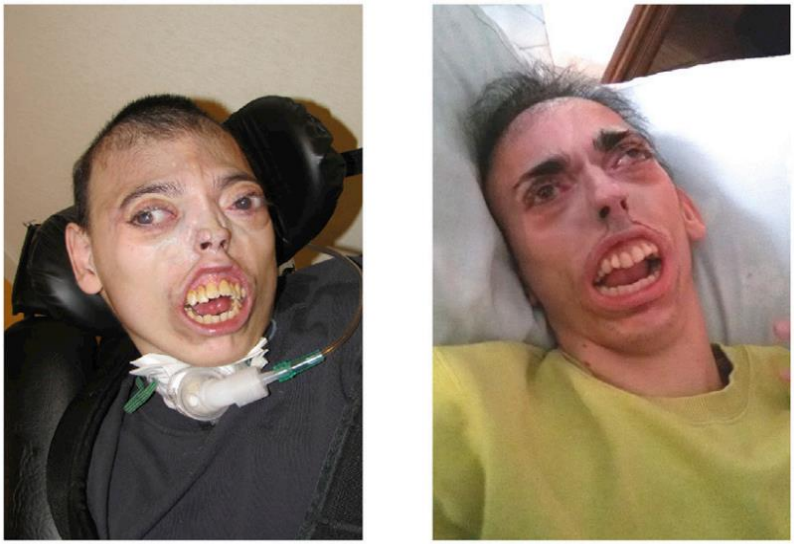


Figure 13: Photographs of two individuals with KCNJ6 mutation c.455_457del (p.Thr152del) (Masotti et al., 2015)

In two patients, three nucleotides (c.455_457del) are deleted, causing the loss of one amino acid, Threonine 152. This affects the protein structure because the channel gets shortened, and the inner base of the selectivity filter is widened. The chains of the subunits are now 10.1 Å apart, instead of 6.9 Å in the wild type. Because of this deletion, the remaining Threonine 151

is shifted towards the bottom of the channel, changing the orientation of Threonine residues that are now directed to the outside of the channel. These changes in conformation can prevent the stabilisation of K^+ ions, interfering with the channel function. (Masotti et al., 2015)

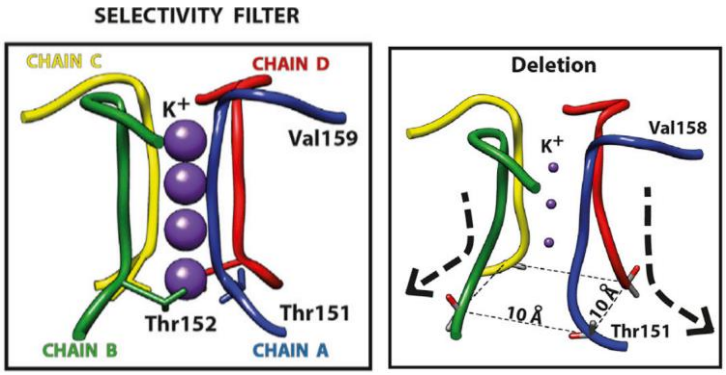


Figure 14: Magnification of the K^+ selectivity filter in the 3SYO protein structure (left)

Deletion of Thr152 shortens the channel and widens the inner base of the selectivity filter (right) (Masotti et al., 2015)

2.2.2. p.Gly154Ser

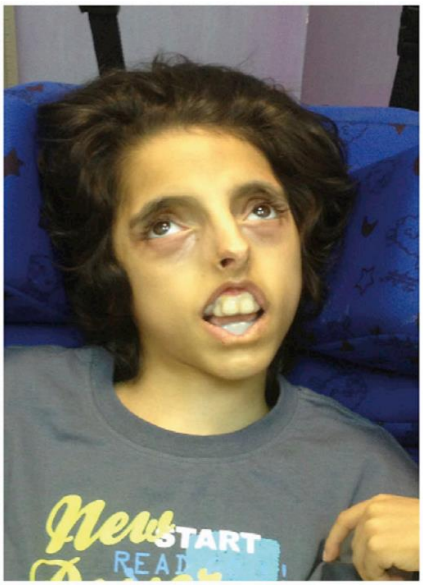


Figure 15: Photograph of an individual with KCNJ6 mutation (p.Gly154Ser) (Masotti et al., 2015)

The missense mutation in the third individual replaces a glycine with a serine located in the middle of the selectivity filter. Serine has more hydrophilic and polar properties than glycine. Their hydroxyl group can lead to inhibition of the ion current or changes in channel gating. This amino acid substitution may also lead to loss of K^+ ion selectivity, causing other cations, like Na^+ to pass through as well. (Masotti et al., 2015)

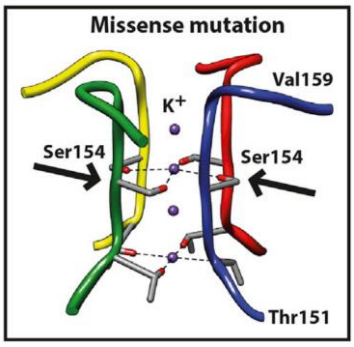


Figure 16: Selectivity filter of the missense mutation (p.Gly154Ser) (Masotti et al., 2015)

2.3. De novo gain-of-function mutation, similar to Keppen-Lubinsky

The fourth individual known with a de novo mutation in KCNJ6 (GIRK2) gene shows symptoms similar to the Keppen-Lubinsky syndrome but without recognisable lipodystrophy or epilepsy. The patient showed severe hyperkinetic movement disorder, developmental delay and hypotonia. (Horvath et al., 2019)

p.Thr152del (near the ion-selective pore)	Two individuals	KPLBS with lipodystrophy
p.Gly154Ser (near the ion-selective pore)	One individual	KPLBS with lipodystrophy
p.Leu171Arg (different region)	One individual	Severe hyperkinetic movement disorder and developmental delay like KPLBS; **without** lipodystrophy

(Horvath et al., 2018)

Whole exome sequencing (WES) was performed for this patient, and it revealed a leucine to arginine missense mutation p.Leu171Arg. In vitro, functional studies were then conducted to find out if this mutation changes the function of GIRK2. These studies showed that expression of the mutant channel alone results in abnormal inward currents that were no longer G-protein-coupled, had lost K^+ selectivity and gained permeability of other cations, like Ca^{2+}. These altered channel characteristics cause sustained depolarisation, instead of the ordinarily inhibitory response of GIRK channels. (Horvath et al., 2018)

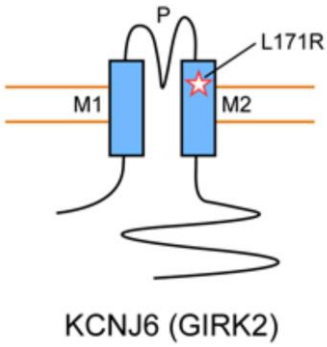


Figure 17: Cartoon of GIRK2 ion channel showing the position of the L171R mutation

P…pore; M1, M2… transmembrane domain (Horvath et al., 2018)

Conformational changes

The leucine to arginine mutation allows arginine to form hydrogen bonds with Glycine148. This electrostatic interaction leads to a more stable structure that limits conformational movements. They prevent the channel from opening or closing and may also impair the ion selectivity. (Horvath et al., 2018)

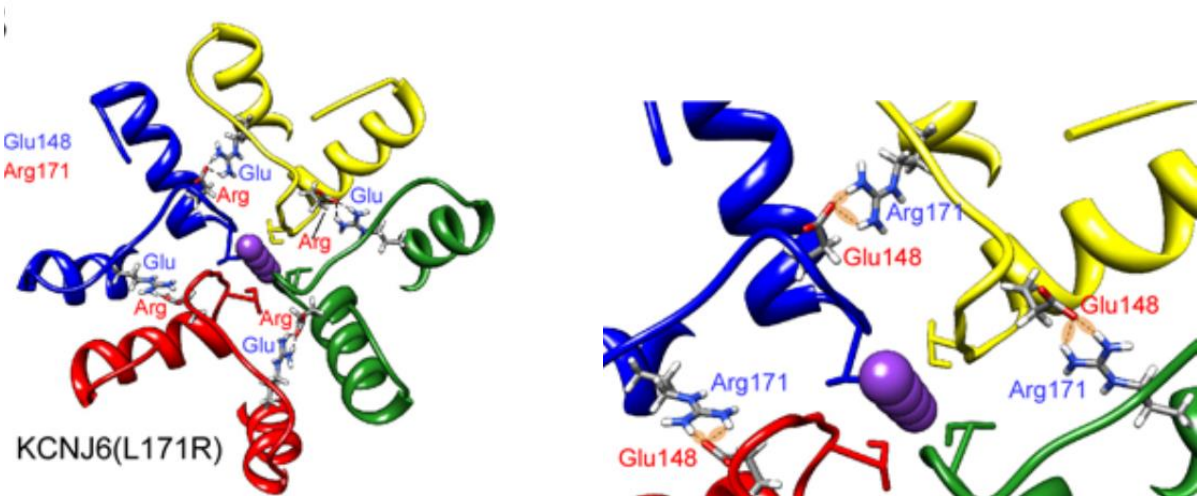


Figure 18: 3D structure of human GIRK2 (PDB: 3SYO),

L171R mutation allows Arg171 to form H-bonds with Glu148 in the subunit next to it. (left) This interaction makes the structure more stable, affects conformational movements and impairs ion selectivity.

3D model showing H-bonds between Arg171 and Glu148 (right) (Horvath et al., 2018)

Horvath et al. describe the case of this four-year-old girl with this point mutation. First concerns arose at the age of 6 months when the baby had no head control, abnormal hand movements and posturing of her legs. At the age of four, dystonic posturing of arms and legs and ballism were observed. She had frequent episodes, in which her eyes rolled up.
EEG (= Electroencephalography) showed background dysrhythmia, but no epileptiform discharges. At eight months, she was diagnosed with developmental delay, hypotonia and the

hyperkinetic movement disorder. Ten months later, they still noticed poor head control, she was unable to sit, did not reach for objects, and she did not crawl. She had feeding difficulties, and that is why she got a feeding tube placed when she was two years old.
First, they tried to treat her with Levodopa, but it led to severe exacerbation of her hyperkinetic movements even with the lowest dose, so they immediately discontinued its usage. They also tried to give her Pramipexole, a dopamine agonist, but she did not tolerate it. With Clonazepam there was minor improvement in her movements, but a side effect was increased salivation. A combination of Trihexylphenidyl (an antiparkinson agent) plus Gabapentin (an anticonvulsant) and tetrabenazine were gradually introduced, and they indeed decreased the severity and frequency of her movements. She still suffers from daily exacerbations, but they are more related to excitement, frustration or anxiety.
If the movements last more than half an hour Clonidine is given, to calm down the movements. (Horvath et al., 2018)

Using site-directed mutagenesis, the single point Leucine-to-Arginine mutation was introduced to the murine GIRK2. Human and mouse GIRK proteins are quite similar, and the homologous leucine in the mouse protein is Leucin173. Human embryonic kidney cells were transfected with different cDNAs, such as the wild type GIRK2 channel, the mutated GIRK2 channel, a wild type GIRK 1 and 2 dimer and a GIRK1 dimer with the mutated GIRK2.

Figure 19: Cartoon to illustrate the similarity between human and mouse GIRK2 protein structure, homologous leucine coloured in red (Horvath et al., 2018)

To measure potassium currents, whole-cell patch-clamp was implemented with an extracellular solution or with different drug solutions.
The drugs used are Baclofen (a $GABA_B$-agonist), propanol (which is a G-protein independent activator), also Bariumchloride (Barium ions are inhibitors) and lastly QX-314 (which is a sodium channel inhibitor). The wild type GIRK 2 channel showed large Baclofen and propanol induced currents that can be inhibited with Barium ions but not with the sodium channel inhibitor QX-314.

In the mutant GIRK channel, G-protein- and alcohol-dependent activation are both impaired, and the channel shows a large agonist-independent basal current. Baclofen, propanol and Barium show little to no inhibition of the channel. On the other hand, QX-314 did inhibit the agonist-independent basal current of this mutated channel.
Co-expression of GIRK1 together with the mutant GIRK2 result in currents similar to the mutant channel on its own, meaning that the heterotetramer either adopts the properties of the mutated channel or that the assembled heterotetramer is not functional. (Horvath et al., 2018)

There is a similar mutation in GIRK2 that also impairs G-protein-activation and is insensitive to Barium, the developmentally impaired weaver mouse. Its depolarising inward current is also inhibited by QX-314.
Despite the difference in species, there are similarities between the clinical phenotype in this present case and the weaver mice. (Horvath et al., 2018)

Weaver mouse mutation

Symptoms of the weaver mutation in mice are severe ataxia, hyperactivity and tremor. Beside the cerebellar defects, homozygote weaver mice also show a progressive loss of dopaminergic neurons similar to the defect in Parkinson's disease.
Perhaps the patient did not tolerate Levodopa, experiencing more severe hyperkinetic movements, due to a degenerating, but overly active dopaminergic network.
This may be the reason for the hypersensitivity to dopaminergic drugs. (Horvath et al., 2018)

3. Methods

3.1. Molecular Docking

Computational modelling and simulation play an essential role in drug discovery. Molecular docking of small molecules against protein binding sites is a structure-based drug design method that was first introduced in the early 1980s. This tool is widely used for hit identification and lead optimisation, as well as for investigations on drug metabolism and toxicity. (B-Rao et al., 2009)

Molecular docking is used to predict interactions between a small molecule and a target protein at an atomic level by computational methods. This can be achieved in two connected actions: at first different binding modes of the ligand in the active site of the receptor are sampled and then ranked by the use of a scoring function. (Meng et al., 2011)

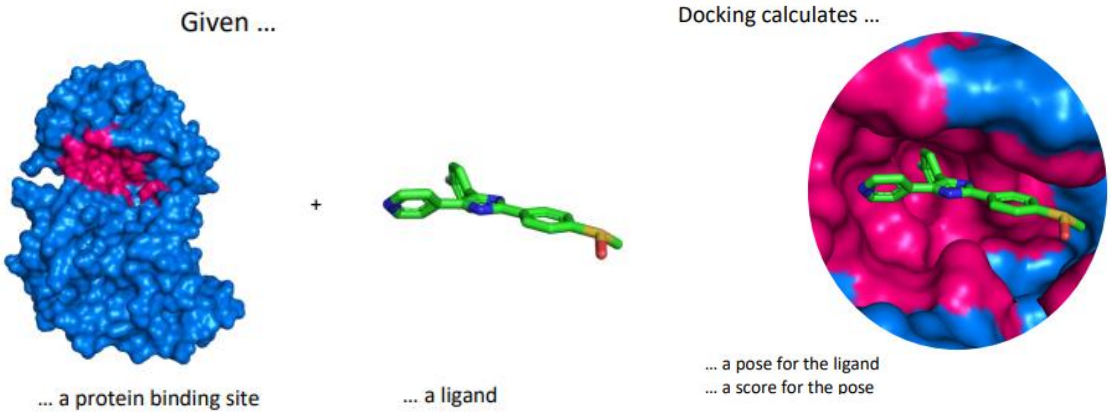


Figure 20: Docking calculates a pose for a ligand and ranks the different conformations by a score. (CCDC, Pose Prediction with GOLD 2020.0 CSD Release)

Because the human genome is relatively well studied nowadays, a large number of new drug targets were found. X-ray, NMR (nuclear magnetic resonance) spectroscopy techniques and homology models contribute to a broader insight into protein structures. These findings make computational strategies able to assert into many aspects of drug discovery. For instance, virtual screening (VS) is a technique for hit identification and lead optimisation, that is more effective, direct and has a lower cost than the traditional high-throughput screening (HTS). (Meng et al., 2011)

Virtual screening is either structure-based or ligand-based. When more information is known about ligand molecules, and there is little to no information about the protein structure, ligand-based methods are used. Examples are QSAR (quantitative structure-activity

relationship) and pharmacophore modelling. Molecular docking, on the other side, is a widespread structure-based method. (Meng et al., 2011)

Docking happens in two stages: the pose (position and orientation of the ligand) is predicted, and then the binding affinity is evaluated.

If the location of the binding site is already known before docking the efficiency is significantly increased. Information about the binding site can be gathered by comparing the target protein with a different protein family with similar functions.

If the binding site is not known online servers or cavity detection programs can be used to determine active sites in the target protein. When no information about the active site is available, blind docking is carried out. (Meng et al., 2011)

The lock-and-key theory by Fischer was the first explanation for the ligand-protein binding mechanism. In early docking methods, the protein and the ligand were both treated as rigid bodies. Koshland later established the "induced-fit" theory, which says that the binding site of the protein is continuously reshaped by the interaction with the ligand, making it a more accurate approach. (Meng et al., 2011) Because flexibility is essential in the binding process, new studies explored ways to view both, the ligand and the protein, as flexible when docking. (B-Rao et al., 2009) However, docking with flexible proteins, especially backbone flexibility, is still the main challenge for existing docking methods. (Meng et al., 2011)

In the last 20 years, over 60 different docking programs have been generated: DOCK, AutoDock, FlexX, GOLD, Glide, LigandFit, FRED, MOE-Dock and many more. (Pagadala et al., 2017)

In molecular docking programs algorithms are designed to evaluate conformations of the ligand until they reach the minimum energy. An affinity scoring function is then utilised to rank the poses. (Pagadala et al., 2017)

Sampling algorithms

There are a great many possible binding poses between ligands and proteins. Designing all these options on the computer would be too expensive. Therefore different sampling algorithms were generated to evaluate these poses. (Meng et al., 2011)

Matching algorithms map the ligand into the binding pocket of the target protein based on molecular shape. Both are represented as pharmacophores, and the distances between them are calculated for a match. Chemical characteristics can be considered in the course of the match. Because matching algorithms are relatively fast, they are used to examine large libraries. (Meng et al., 2011)

Incremental constructions methods position the ligand fragmentally into the binding site. The rotatable bonds of the ligand get broken and one of the fragments, usually the largest, is chosen to dock into the binding site and the other pieces are added gradually so that different orientations are generated. (Meng et al., 2011)

Stochastic methods randomly adjust the ligand conformation. Monte Carlo and genetic algorithms both belong to this class. (Meng et al., 2011)
The conformation obtained by the Monte Carlo method is examined by energy-based selection standards. Poses that correspond to these standards are further modified to design the next conformation. This is repeated until the required quantity of conformations is reached. An advantage of the Monte Carlo method over molecular dynamics simulations is that modifications can be quite large so that energy barriers can be exceeded. (Meng et al., 2011)
Genetic algorithms are a common stochastic method, whose idea comes from Darwin's theory of evolution. "Genes" in this method are the degrees of freedom of the ligand, that are translated into binary strings. Many of these "genes" together constitute the "chromosome", which results in the pose of a ligand. Genetic algorithms are used in programs like GOLD, AutoDock and DARWIN. (Meng et al., 2011)

Molecular dynamics (MD) is a powerful simulation method, commonly used in computational modelling. Flexibility of the protein and the ligand are better represented in MD simulations than other algorithms because each atom is moved separately in the field. The drawback of MD simulations is that they work in relatively small steps, making it more challenging to overcome the high energy conformational barriers. However, MD simulations are very useful in local optimisation. (Meng et al., 2011)

Scoring functions

The aim of a scoring function is to distinguish correct from incorrect poses. They estimate, rather than calculate binding affinities between the target and ligand. Scoring functions can be force-field-based, empirical or knowledge-based. (Meng et al., 2011)

Force-field-based scoring functions evaluate the binding energy by determining the sum of electrostatic and van der Waals interactions. These interactions are calculated with a Coulombic formula. (Meng et al., 2011)

Extensions of this scoring function also take hydrogen bonds, solvations and entropy contributions into account. Software programs, like DOCK, GOLD and AutoDock, offer this scoring function. (Meng et al., 2011)

Empirical scoring functions divide the binding energy into different energy components, like ionic interactions, hydrogen bonds, the hydrophobic effect and binding entropy. To get a final score, each of these components is multiplied by a coefficient and summed up. (Meng et al., 2011) Energy terms in empirical scoring functions are quite easy to evaluate, but they are not always well suited for ligand-protein complexes other than the training set. LUDI, PLP and ChemScore [83] are examples for programs offering empirical scoring functions. (Meng et al., 2011)

Knowledge-based scoring functions try to get information about interatomic contact frequencies and distances between the target protein and the ligand, by using statistical analysis of the crystal structures. This knowledge is based on the hypothesis that more favourable interactions occur with higher frequency. These frequency distributions are then translated into pairwise atom-type potentials. (Meng et al., 2011)

The advantage of knowledge-based functions is its computational simplicity, which can be applied for screening large ligand databases. Nevertheless, it is still a problem that some interactions are not represented in the incomplete training sets of crystal structures. (Meng et al., 2011)

The scoring function is an essential element of molecular docking that needs to be further improved. Experimental technology is still required to get realistic interactions between ligands and proteins. (Meng et al., 2011)

3.2. Description of used programs

3.2.1. GOLD

Genetic Optimisation for Ligand Docking (GOLD) is a widely used automated docking program published in 1995. The program is genetic algorithm based with flexible ligands and partial flexibility for protein. It is a collaboration between CCDC (Cambridge Crystallographic Data Centre), GlaxoSmithKline plc and the University of Sheffield. (Jones et al., 1997)

GOLD's success rate in docking a ligand back to its experimentally observed binding site is relatively high (71%). (Jones et al., 1997)
Further analyses of these results were carried out to determine why the docking process may fail and what makes it succeed. Examination of ligand composition showed that the algorithm most likely fails when the ligand is hydrophobic or when the protein binding site is not resolved well. GOLD does not have an efficient mechanism to sample hydrophobic interactions. Also, the genetic algorithm scoring function does not involve terms for desolvation. Other interactions that are not considered by the scoring function are: contacts between groups that are rich in electrons and groups that are electron-deficient; non-bonded interactions; ring stacking and hydrogen bonds to pi systems. An extension of the algorithm to include these contacts is pursued. (Jones et al., 1997)

A downside of the algorithm is that it requires quite a lot of time. (Jones et al., 1997)
A more significant limitation of the algorithm is that the protein is basically rigid. This is not a problem for systems, where the conformational changes of the binding site, between the bound and unbound complex, are small enough. (Jones et al., 1997)

Genetic algorithm mimics the process of evolution by managing a set of data structures, referred to as chromosomes. Possible solutions (poses) are translated into these structures and can be assigned a score. (Jones et al., 1997) Accuracy is mostly affected by the number of dockings and genetic algorithm operations in each docking. (Verdonk et al., 2003)

3.2.2. LigandScout

LigandScout is an innovative molecular modelling and design software that consists of a graphical desktop application and command-line tools. A major advantage of LigandScout is the lower costs in the initial stages of drug discovery, making in silico experiments possible. (Kainrad et al., 2019)

In the last decades, pharmacophore modelling has become an essential and effective method for drug discovery. It is crucial for describing molecules and complexes, such as proteins or nucleic acids. Describing drug interactions with pharmacophore models is a common and reasonable method for high-throughput virtual screening. (Wolber et al., 2005)

A pharmacophore model consists of chemical functionalities aligned in a three-dimensional room. This composition of chemical characteristics represents the main interactions of small molecules with a macromolecular protein target. As a result, a pharmacophore model just represents a single mode of action. (Wolber et al., 2005)

While virtual screening cannot compete with experimental screening regarding false-negative and false-positive rates, VS can process far more extensive compound databases. (Kainrad et al., 2019)

Figure 1

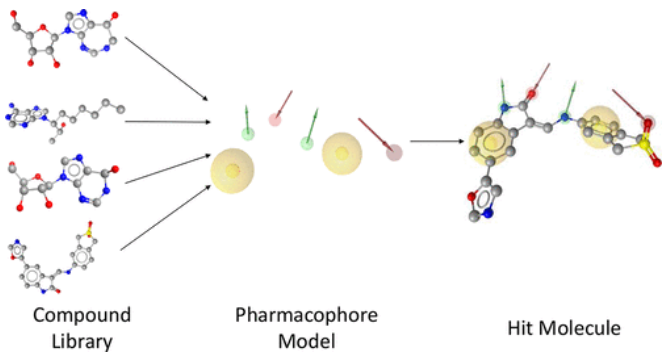


Figure 21: Virtual screening by use of pharmacophore models. In the virtual screening process, input compounds are examined for how well they fit the desired pharmacophores. (Kainrad et al., 2019)

LigandScout was applied in this diploma study to create pharmacophore models of each compound in its most favourable binding position.

3.2.3. Avogadro

Avogadro is a molecule visualiser and editor generated for cross-platform use in computational modelling and other related areas. Avogadro is often used to build molecular structures, format input files and analyse output files of many other computational programs. (Hanwell et al., 2012)

Avogadro is a user-friendly builder with integrated support for transferring structures from databases like PubChem and the Protein Data Bank, obtaining chemical data in various formats. By an efficient plugin mechanism, Avogadro can easily be extended for developers, to provide additional features. (Hanwell et al., 2012)

In this diploma study, Avogadro was used for building the six compounds (XE991, M084, ML204, ML297, RTG and RL648-81), that were later docked to the GIRK2 protein (PDB: 3SYA and 4KFM).

4. Aims

This diploma study aims to find binding sites for potential inhibitors for the de novo gain-of-function mutation p.Leu171Arg in KCNJ6 (GIRK2) genes. This mutation makes the channel lose its K^+ selectivity causing abnormal inward currents that are no longer G-protein-coupled. These changed channel properties cause sustained depolarisation, instead of the normally inhibitory response of GIRK channels. (Horvath et al., 2018) Blocking these mutated channels could potentially prevent this inward current of cations, that causes depolarisation and is responsible for the symptoms in the patient. Symptoms are similar to these in Keppen-Lubinsky syndrome, but without recognisable lipodystrophy or epilepsy. The individual shows severe hyperkinetic movement disorder, developmental delay and hypotonia. (Horvath et al., 2018)

A recent study, published in 2020, determined that the following six compounds (XE991, M084, M204, ML297, RTG and RL648-81) block GIRK2. (De la Rosa and Shapiro, 2020) These six compounds were docked against two crystal structures of GIRK2, 3SYA, a structure of GIRK2 in complex with sodium and PIP2 (Whorton et al., 2011), and 4KFM, a structure of GIRK2 in complex with the Gβγ subunits (Whorton et al., 2013).

This was done to find appropriate binding sites for each of the compounds and rank them according to their binding affinity.
However, experimental studies need to be conducted, to examine further, if these compounds would actually bind to the disease mutant.

5. Results

5.1. Protein structures

5.1.1. 3SYA

Crystal structure of GIRK2 (Kir3.2) in complex with Na^+ and PIP2

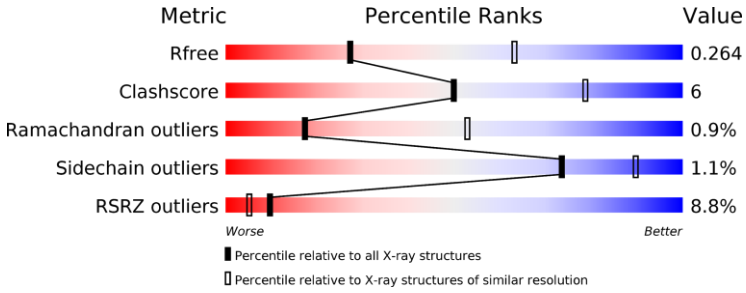
Classification: METAL TRANSPORT
Organism(s): Mus musculus
Expression System: Komagataella pastoris
Mutation(s): No

Deposited: 2011-07-16 **Released:** 2011-10-12
Deposition Author(s): Whorton, M.R., MacKinnon, R.

https://www.rcsb.org/structure/3SYA

Experimental Data Snapshot

Method: X-RAY DIFFRACTION
Resolution: 2.98 Å
R-Value Free: 0.269
R-Value Work: 0.240
R-Value Observed: 0.242



https://www.rcsb.org/structure/3SYA

GIRK channels regulate electrical excitability in various cells of the body. They, among other things, control the heart rate and many neuronal activities. 3SYA is the first crystal structure of a GIRK channel presented. (Whorton et al., 2011) Whorton et al. generated a 2.98 Å resolution crystal structure of the GIRK2 channel bound to Na^+ and PIP2.

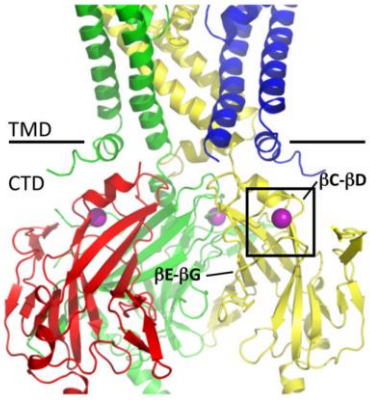


Figure 22: Na^+ binding site in GIRK2 lies between the βC-βD and βE-βG loops and is dependent on the negatively charged Asp228. Subunits are displayed in different colours, and the front subunit is not shown for clarity. Na+ ions are displayed in purple. (Whorton et al., 2011)

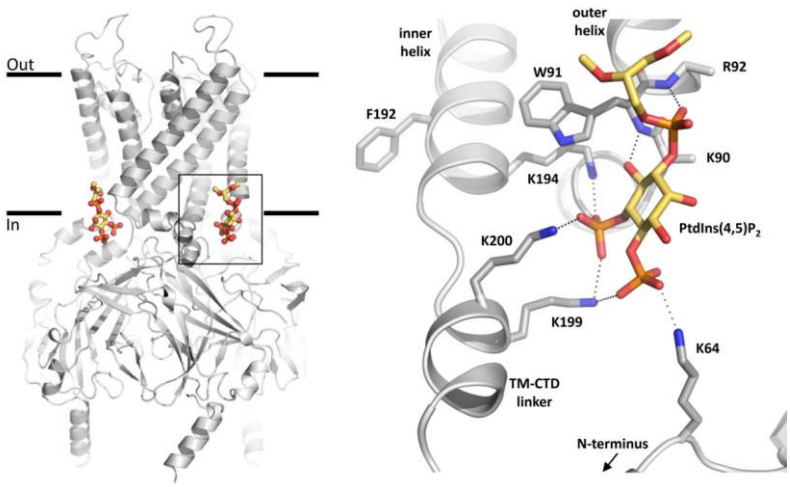


Figure 23: PIP2 bound to a GIRK channel (left) and a close-up view (right). PIP2 is located at the interface between the transmembrane and cytosolic domains. It is mainly directed by the positively charged residues in GIRK. (Whorton and MacKinnon, 2011)

Topology

Feature key	Position(s)	Description	Graphical view	Length
Topological domain	1 – 91	Cytoplasmic By similarity		91
Transmembrane	92 – 116	Helical; Name=M1 By similarity		25
Topological domain	117 – 140	Extracellular By similarity		24
Intramembrane	141 – 152	Helical; Pore-forming; Name=H5 By similarity		12
Intramembrane	153 – 159	Pore-forming By similarity		7
Topological domain	160 – 168	Extracellular By similarity		9
Transmembrane	169 – 190	Helical; Name=M2 By similarity		22
Topological domain	191 – 425	Cytoplasmic By similarity		235

Molecule processing

Feature key	Position(s)	Description	Graphical view	Length
Chain (PRO_0000154944)	1 – 425	G protein-activated inward rectifier potassium channel 2		425

Amino acid modifications

Feature key	Position(s)	Description	Graphical view	Length
Modified residue	18	Phosphoserine Combined sources		1
Modified residue	25	Phosphoserine Combined sources		1

Motif

Feature key	Position(s)	Description	Graphical view	Length
Motif	154 – 159	Selectivity filter By similarity		6
Motif	422 – 425	PDZ-binding		4

https://www.uniprot.org/uniprot/P48542

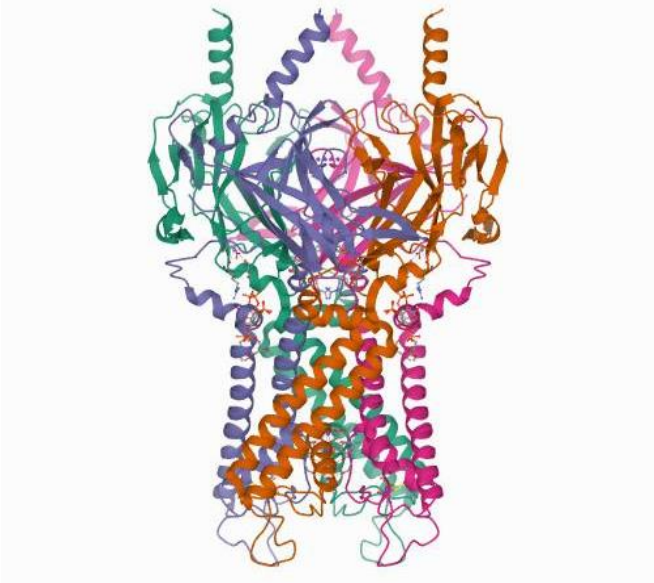


Figure 24: Crystal structure of GIRK2 in complex with sodium and PIP2 (PDB: 3SYA)

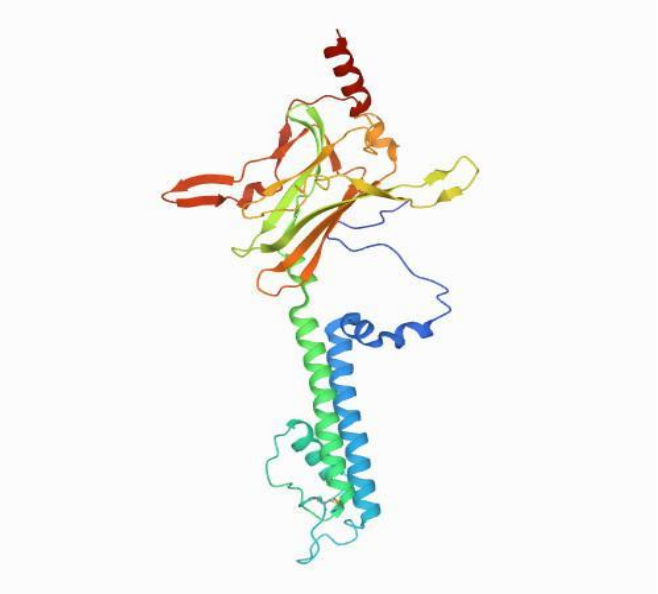


Figure 25: Asymmetric unit of GIRK2 (PDB: 3SYA)

5.1.2. 4KFM

Crystal structure of GIRK2 (Kir3.2) in complex with the Gβγ protein subunits

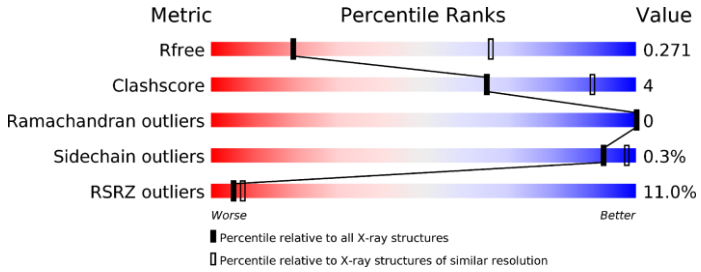
Classification: METAL TRANSPORT
Organism(s): Mus musculus, Homo sapiens
Expression System: Komagataella pastoris, Spodoptera frugiperda
Mutation(s): No

Deposited: 2013-04-27 **Released:** 2013-06-12
Deposition Author(s): Whorton, M.R., MacKinnon, R.

https://www.rcsb.org/structure/4KFM

Experimental Data Snapshot

Method: X-RAY DIFFRACTION
Resolution: 3.45 Å
R-Value Free: 0.265
R-Value Work: 0.228
R-Value Observed: 0.230



https://www.rcsb.org/structure/4KFM

Through G-protein-coupled receptor stimulation, GIRK channels allow neurotransmitters to regulate the electrical excitability of cells. Whorton et al. generated a 3.45 Å resolution crystal structure of the mammalian GIRK2 channel bound to Gβγ protein subunits.
Four Gβγ protein subunits are stabilised by short-range atomic and wide-range electrostatic interactions, leading to a pre-open state of GIRK2. This state is a conformation that happens between the open and the closed conformation of the constitutively active mutant.
This 4KFM structure gives a theoretical insight into how the signalling lipid PIP2, together with intracellular Na^+ ions contribute to multi-ligand regulation of GIRK2 channels.
(Whorton et al., 2013)

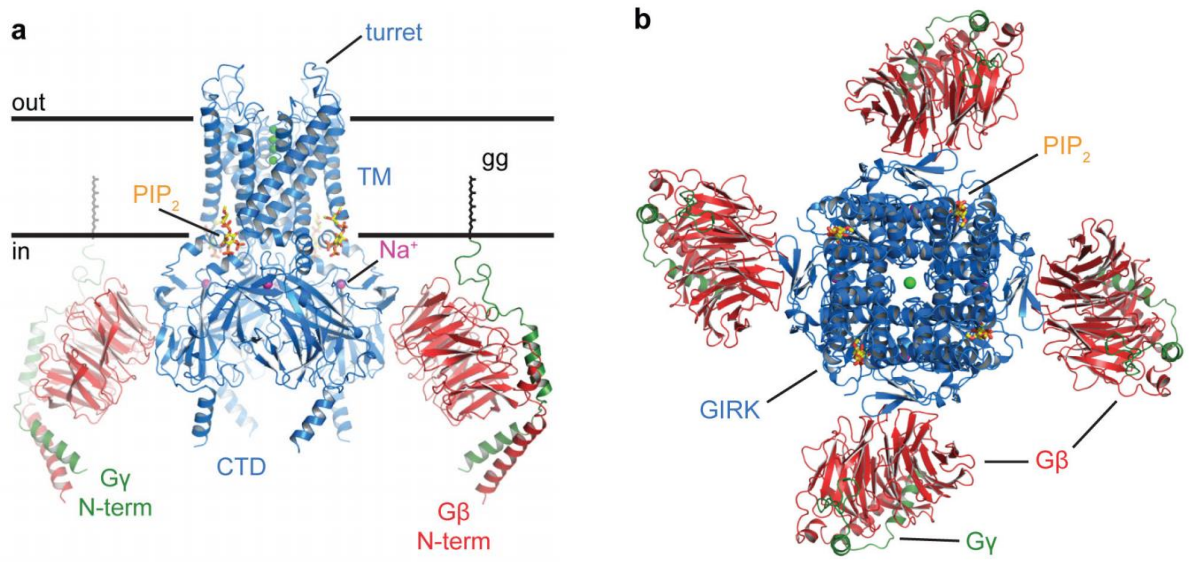


Figure 26: A side (left) and top-down view from the extracellular side (right) of the complex. The front Gβγ dimer is not shown for clarity. The phospholipid bilayer is demonstrated by the black lines. Na+ ions are illustrated as purple spheres. Gβ and Gγ proteins are coloured, red and green. PIP2 molecules are displayed as sticks. K+ ions are shown in green. (Whorton et al., 2013)

Topology

Feature key	Position(s)	Description	Graphical view	Length
Topological domain	1 – 91	Cytoplasmic By similarity		91
Transmembrane	92 – 116	Helical; Name=M1 By similarity		25
Topological domain	117 – 140	Extracellular By similarity		24
Intramembrane	141 – 152	Helical; Pore-forming; Name=H5 By similarity		12
Intramembrane	153 – 159	Pore-forming By similarity		7
Topological domain	160 – 168	Extracellular By similarity		9
Transmembrane	169 – 190	Helical; Name=M2 By similarity		22
Topological domain	191 – 425	Cytoplasmic By similarity		235

Molecule processing

Feature key	Position(s)	Description	Graphical view	Length
Chain (PRO_0000154944)	1 – 425	G protein-activated inward rectifier potassium channel 2		425

Amino acid modifications

Feature key	Position(s)	Description	Graphical view	Length
Modified residue	18	Phosphoserine Combined sources		1
Modified residue	25	Phosphoserine Combined sources		1

Motif

Feature key	Position(s)	Description	Graphical view	Length
Motif	154 – 159	Selectivity filter By similarity		6
Motif	422 – 425	PDZ-binding		4

https://www.uniprot.org/uniprot/P48542

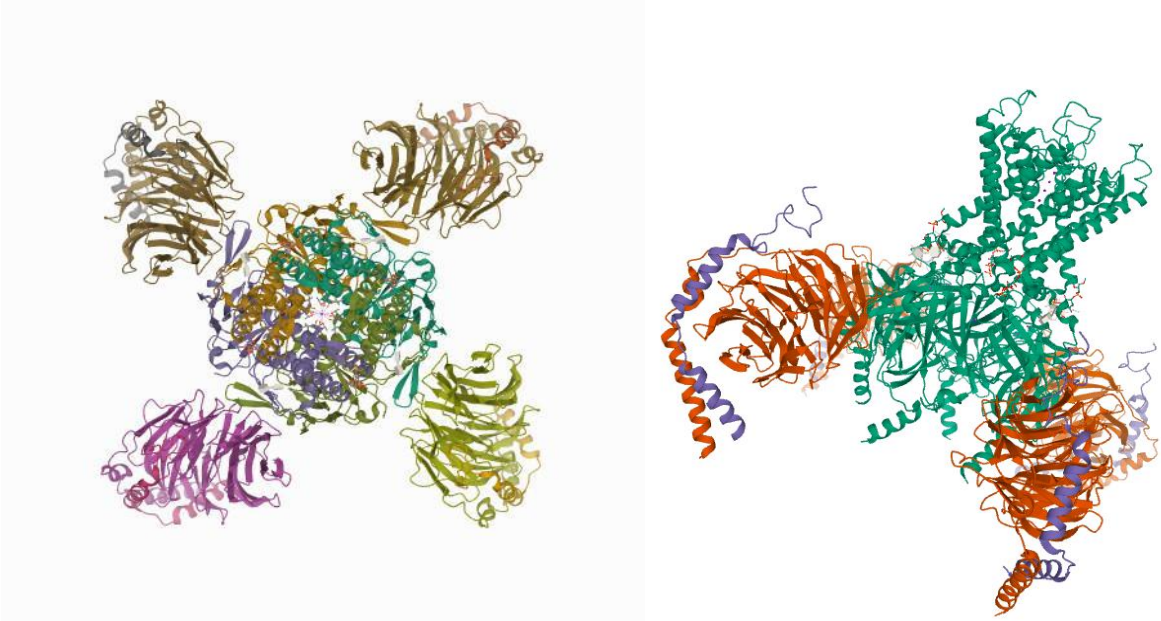


Figure 27: Crystal structure of GIRK2 in complex with four Gβγ protein subunits (PDB: 4KFM)

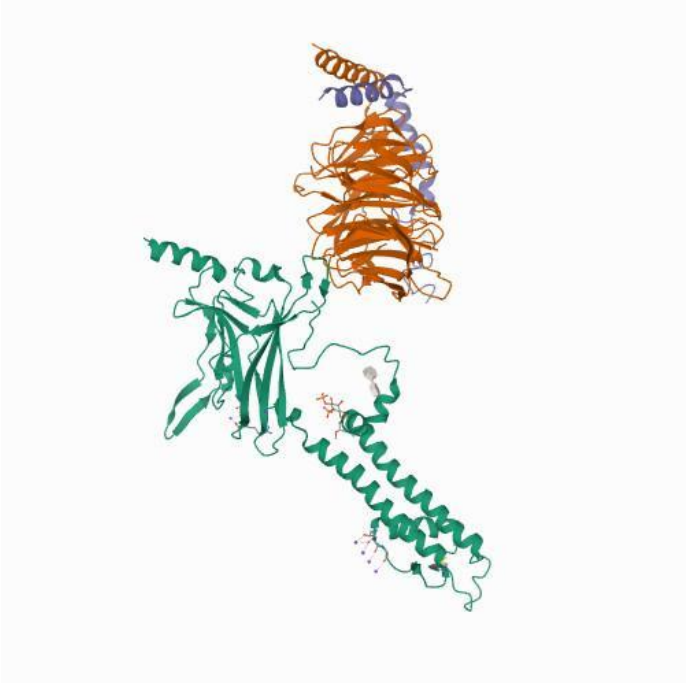
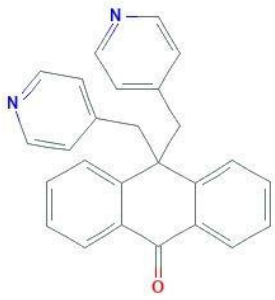


Figure 28: Asymmetric unit of GIRK2 (PDB: 4KFM)

5.2. Compounds

5.2.1. XE991

The compound



$C_{26}H_{20}N_2O$

10,10-Bis(pyridin-4-ylmethyl)anthracen-9-one

Molecular Weight	376.4 g/mol
Hydrogen Bond Donor Count	0
Hydrogen Bond Acceptor Count	3
Rotatable Bond Count	4
Topological Polar Surface Area	42.8 Å²
Covalently Bonded Unit Count	1

https://pubchem.ncbi.nlm.nih.gov/compound/656732

Use of the compound

XE991 is a M-channel inhibitor, which is state-dependent and prefers the activated subunit of Kv7 (KCNQ) channels in nerve cells. (Greene et al., 2017)

M-type channels are voltage-gated and found throughout the nervous system where they play essential roles (Schroeder et al., 2000)

The M-channel is a potassium channel which is slowly activating and involved in managing neuronal excitability. The channel protein is encoded by the KCNQ2 gene and it assembles with another protein encoded by the KCNQ3 gene, the two of them being integral membrane proteins. Defects in channel functioning can lead to a particular type of epilepsy. (Yum MS et al., 2010)

Inhibition kinetic analysis indicates that XE991 binds to a single activated subunit rather than an open channel.

The inhibitor is not potent around the resting potential of cells, making it not efficacious for silent or barely firing neurons. Inhibition is affiliated with channel activation and is confirmed to be voltage dependent. Studies suggest that changes in efficacy are due to conformational changes of the Kv7.2 channel subunits, not voltage changes throughout the plasma membrane. Inhibition of Kv7.2 channels by XE991 is not reversible but can be restored by channel trafficking. (Greene et al., 2017)

Binding Site

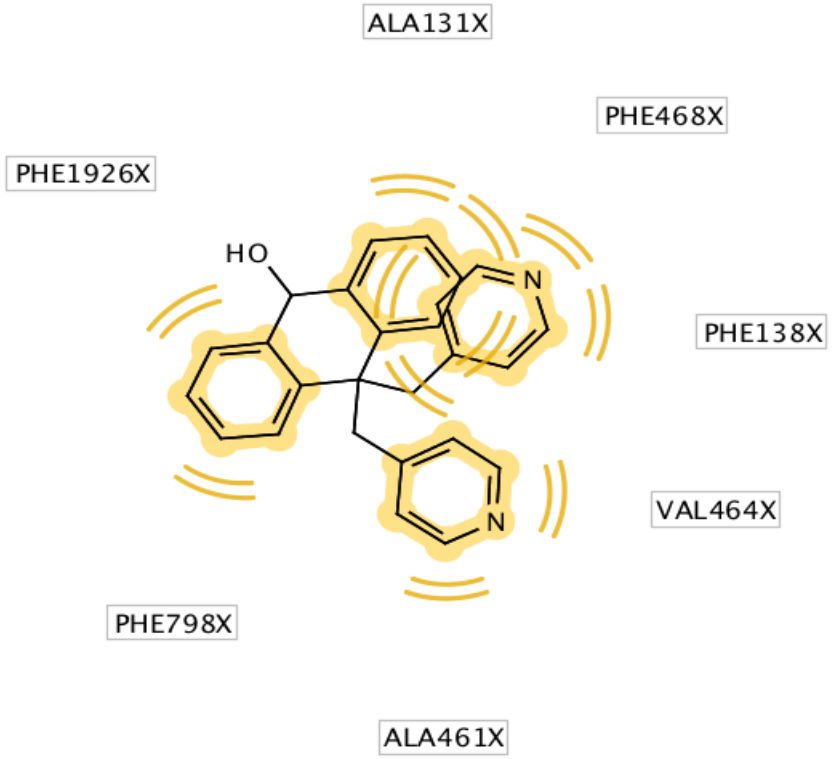


Figure 29: Pharmacophore model of XE991 bound to GIRK2 (PDB: 4KFM)

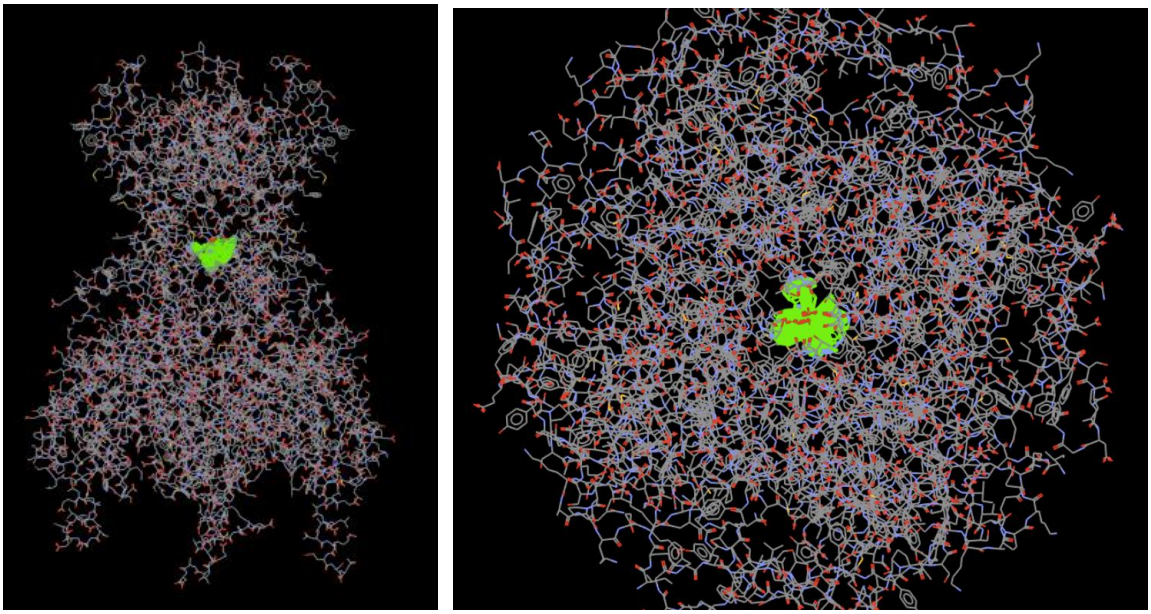


Figure 30: A side (left) and top-down (right) view of XE991 bound to the transmembrane region of GIRK2 (PDB: 4KFM).

The most favourable position for the compound XE991 is predicted to be in the central transmembrane cavity of the 4KFM structure. The Estimated Affinity of this binding pose is high (> μM), and the compound blocks the pore almost in the middle.

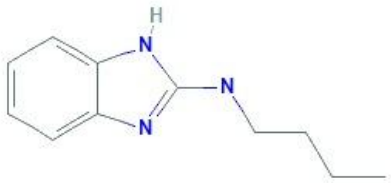
The pharmacophore created with LigandScout showed that the four aromatic rings of the compound form hydrophobic interactions with the hydrophobic amino acids Phe138, Phe468, Phe798 and Phe1926.

How to validate this hypothesis?

By classical mutagenesis, it can be experimentally tested if XE991 would bind to the disease mutant. For this purpose, the hydrophobic amino acid phenylalanine can be changed to threonine. Threonine is a polar amino acid that is no longer able to form hydrophobic interactions, which are essential for the binding of XE991.

5.2.2 M084

The compound



$C_{11}H_{15}N_3$

N-butyl-1H-benzimidazol-2-amine

Molecular Weight	189.26 g/mol
Hydrogen Bond Donor Count	2
Hydrogen Bond Acceptor Count	2
Rotatable Bond Count	4
Topological Polar Surface Area	40.7 Å²
Covalently Bonded Unit Count	1

https://pubchem.ncbi.nlm.nih.gov/compound/n-Butyl-1h-benzimidazol-2-amine

Use of the compound

M084 is potent in inhibiting TRPC4 and TRPC5 and weak in inhibition of TRPC3. (Zhu et al., 2015) TRPC (Transient receptor potential canonical) channels are non-selective cation channels, which are permeable for Ca^{2+} ions. TRPC channels play essential roles in various physiological processes, such as smooth muscle contractions and synaptic conduction, and they are involved in many diseases. (Zhu et al., 2015)

TRPC channels are extensively expressed in the brain, and they are engaged in several functions of the brain. TRPC4 and TRPC5 have both been involved in innate fear function, which is a significant response to environmental stress. But it is still not known how much these channels are implicated in psychiatric diseases. However, the antidepressant and anxiolytic-like effects of the novel TRPC4/C5 inhibitor, M084 have been proven in mice. (Yang et al., 2015)

Because there is a lack of potent type-specific inhibitors, the role of TRPC channels, up till now, could not be elucidated well. (Zhu et al., 2015)

Binding Site

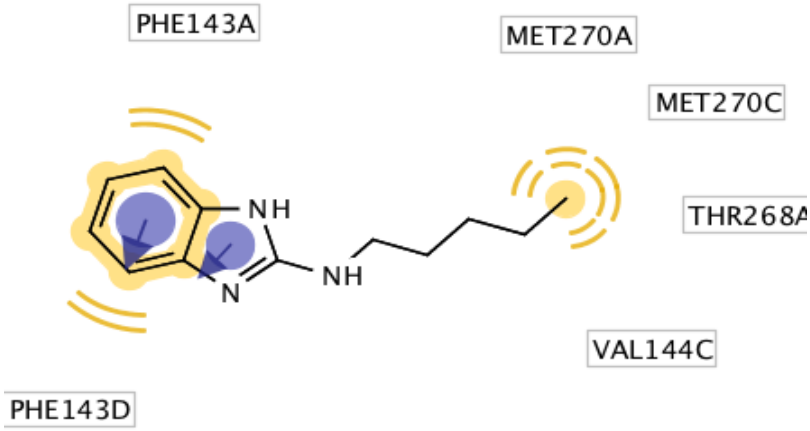


Figure 31: Pharmacophore model of M084 bound to GIRK2 (PDB: 3SYA)

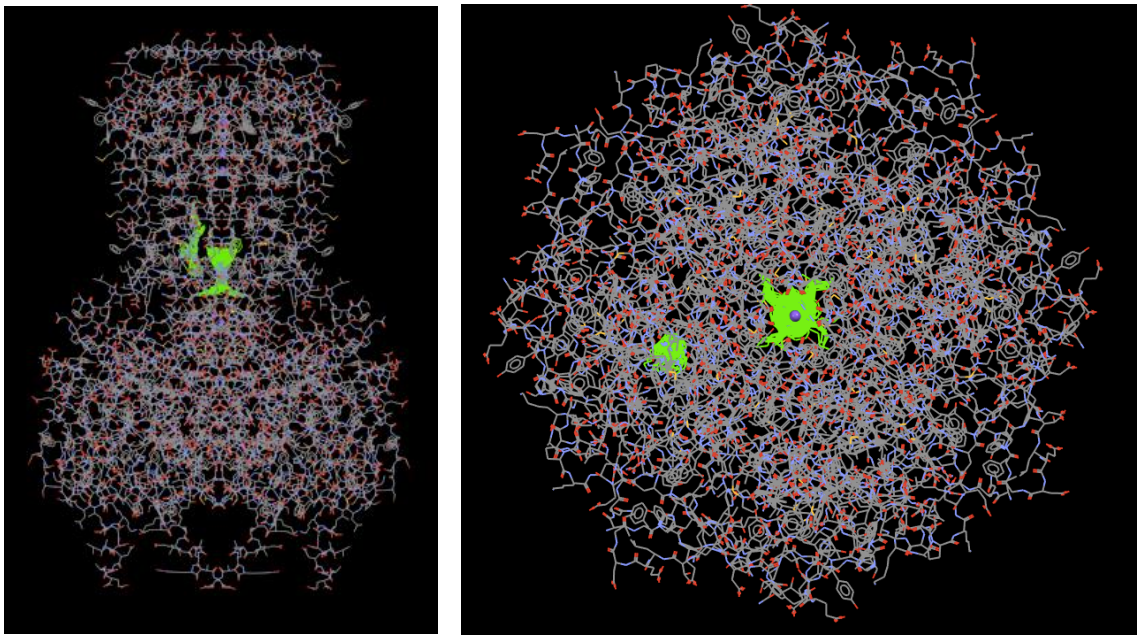


Figure 32: A side (left) and top-down (right) view of M084 bound to the transmembrane region of GIRK2 (PDB: 3SYA).

M084 is predicted to have its best Affinity in the central transmembrane cavity of the narrower 3SYA structure. The Binding Affinity Score lies between mM and low µM and the compound docks in two main clusters, one inside the pore and the other one is shifted to the outside.

The aromatic rings of the structure form pi-pi molecular interactions with the aromatic amino acid Phe143. The alkyl chain forms hydrophobic interactions with the hydrophobic amino acids Met270 and Val144.

How to validate this hypothesis?

To test if M084 binds to the disease mutant classical mutagenesis can be implemented. The aromatic amino acid phenylalanine can be changed to alanine to avoid pi-pi interactions. Alanine is a hydrophobic, non-polar amino acid, without the aromatic ring in phenylalanine.

The hydrophobic amino acids methionine and valine can be mutated to a polar amino acid like threonine so that no hydrophobic interactions can happen between them and the hydrophobic alkyl chain in M084.

5.2.3. ML204

The compound

$C_{15}H_{18}N_2$

4-Methyl-2-(piperidine-1-yl)quinoline

Molecular Weight	226.32 g/mol
Hydrogen Bond Donor Count	0
Hydrogen Bond Acceptor Count	2
Rotatable Bond Count	1
Topological Polar Surface Area	16.1 Å²
Covalently Bonded Unit Count	1

https://pubchem.ncbi.nlm.nih.gov/compound/230710

Use of the compound

ML204 is reported to be a novel and potent channel inhibitor, selective for TRPC4 channels. Whole-cell patch-clamp experiments showed that ML204 blocked TRPC4β currents and indicated a direct interaction of the compound with TRPC4 channels rather than interference with transduction pathways.

ML204 is a potent novel tool for analysing TRPC4 channel function and may help in the development of drugs that target TRPC4 channels. (Miller et al., 2011)

Binding Site

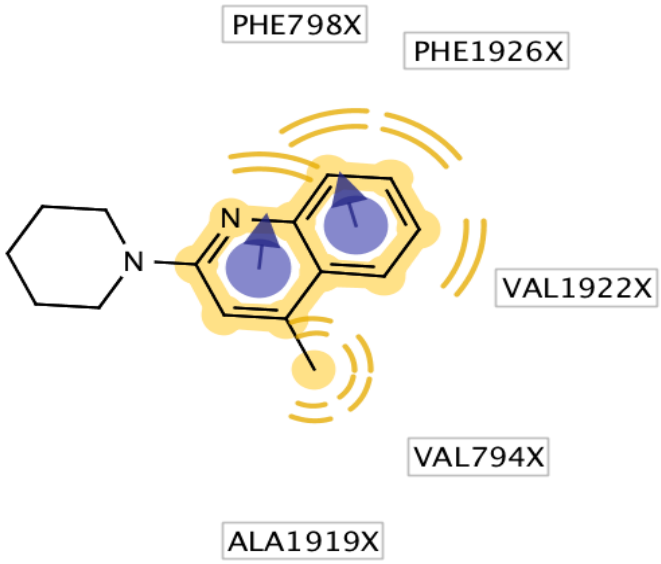


Figure 33: Pharmacophore model of ML204 bound to GIRK2 (PDB: 4KFM)

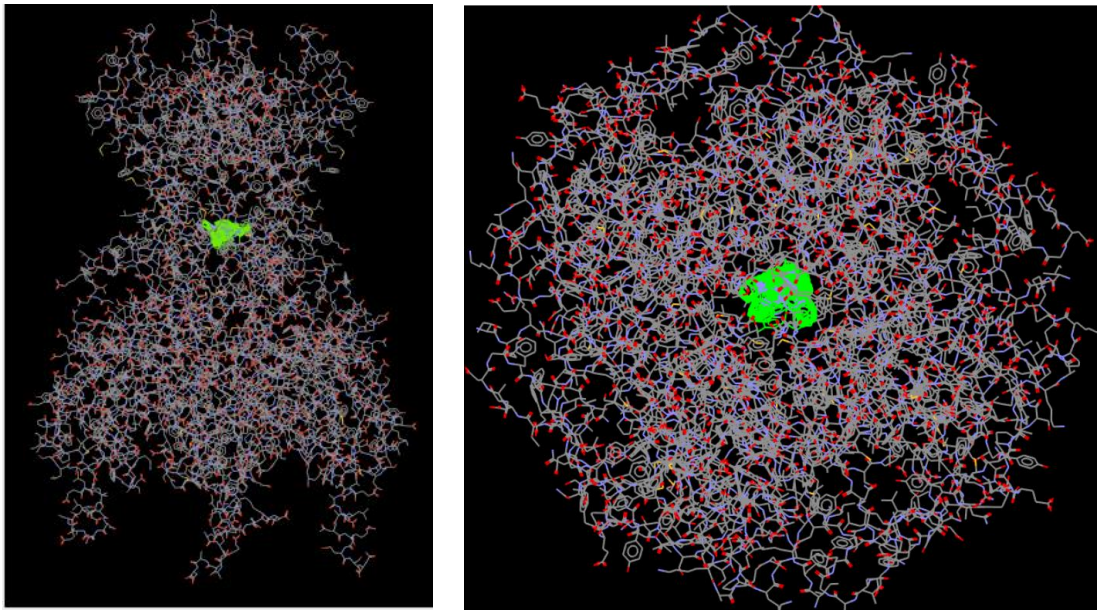


Figure 34: A side (left) and top-down (right) view of ML204 bound to the transmembrane region of GIRK2 (PDB: 4KFM).

The most favourable binding position for ML204 is predicted to be in the central transmembrane cavity of the 4KFM structure. The compound docks in the middle of the pore and the Estimated Affinity is below μM.

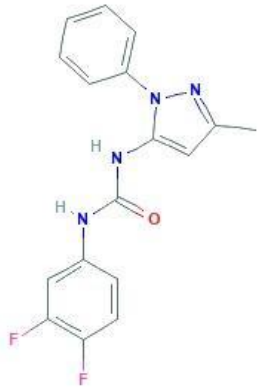
Both of the aromatic rings of the structure form pi-pi molecular interactions with Phe798 and Phe1926, and they form hydrophobic interactions with the amino acids Ala1919, Val794, Val1922, Phe798 and Phe1926.

How to validate this hypothesis?

This hypothesis can be experimentally proven via classical mutagenesis. Phenylalanine can be mutated to alanine, to disturb the pi-pi interactions or the hydrophobic amino acid valine can be changed into polar threonine.

5.2.4. ML297

The compound



$C_{17}H_{14}F_2N_4O$

1-(3,4-Difluorophenyl)-3-(3-methyl-1-phenyl-1H-pyrazol-5-yl)urea

Molecular Weight	328.32 g/mol
Hydrogen Bond Donor Count	2
Hydrogen Bond Acceptor Count	4
Rotatable Bond Count	3
Topological Polar Surface Area	59 Å²
Covalently Bonded Unit Count	1

https://pubchem.ncbi.nlm.nih.gov/compound/56642816

Use of the compound

ML297 is the first potent and selective GIRK activator. The compound is able to activate GIRK1/3 and GIRK1/4 assemblies, but it was unable to regulate HEK-293 cells expressing GIRK2 alone or assembled as GIRK2/3 complexes. It seems that ML297 is just able to activate GIRK channels with a GIRK1 subunit, supporting the hypothesis that a part of the ML297 binding site is exclusive to GIRK1. Because the ability of ML297 is restricted to GIRK channels with a GIRK1 subunit, this compound presents an opportunity to investigate further the roles of GIRK channels with and without GIRK1 subunits. Further study is needed to understand the connection between G-protein-gated regulation and the channel's activation by compounds like ML297. (Kaufmann et al., 2013)

Binding Site

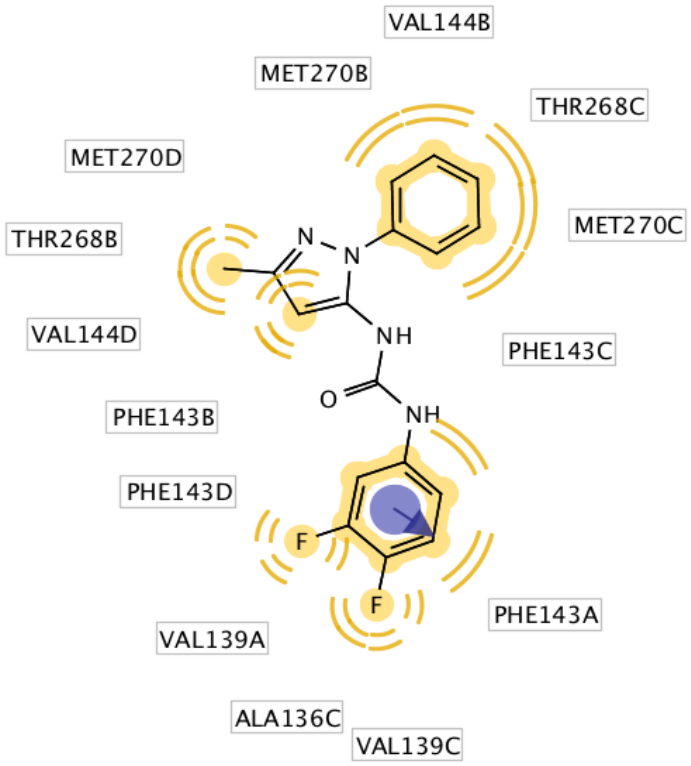


Figure 35: Pharmacophore model of ML297 bound to GIRK2 (PDB: 3SYA)

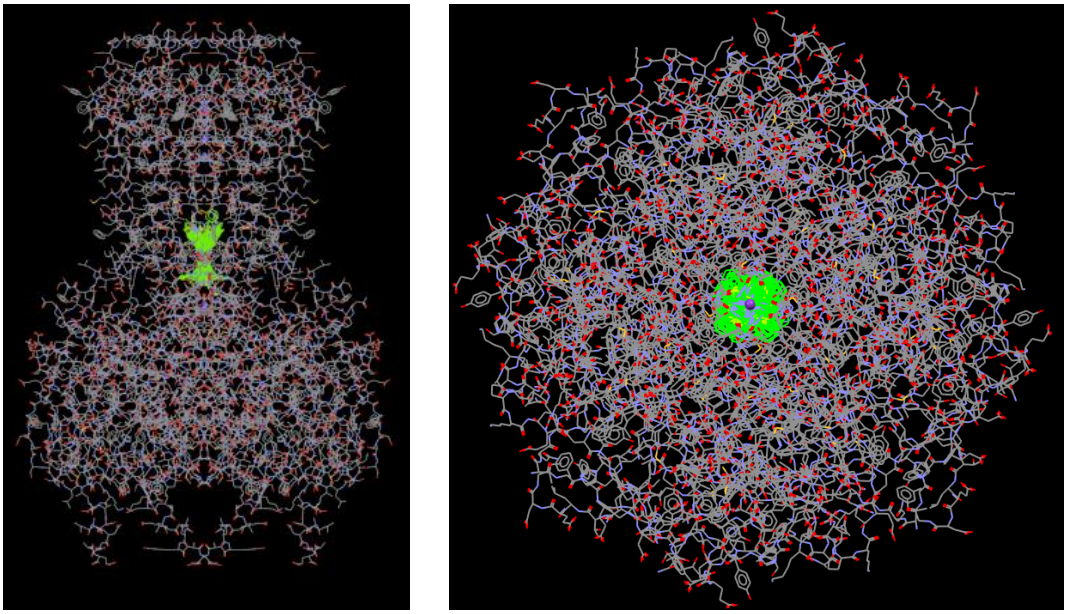


Figure 36: A side (left) and top-down (right) view of ML297 bound to the transmembrane region of GIRK2 (PDB: 3SYA).

With a high Estimated Affinity (> μM) the compound ML297 is predicted to bind in the central transmembrane cavity of the 3SYA structure. It blocks the pore perfectly in the middle and therefore seems to be a promising inhibitor.
One of the aromatic rings in the structure forms pi-pi molecular interactions with the aromatic amino acid Phe143. The many other hydrophobic parts form hydrophobic interactions with the amino acids Val144, Val139, Ala136 and Met270.

How to validate this hypothesis?
Experimentally this hypothesis can be proven by classical mutagenesis. Alanine can be mutated into the polar amino acid threonine, to prevent hydrophobic interactions, and phenylalanine can be changed into alanine, an amino acid that is no longer aromatic.

5.2.5. Retigabine RTG

The compound

$C_{16}H_{18}FN_3O_2$

Retigabine

Molecular Weight	303.33 g/mol
Hydrogen Bond Donor Count	3
Hydrogen Bond Acceptor Count	5
Rotatable Bond Count	6
Topological Polar Surface Area	76.4 Å²
Covalently Bonded Unit Count	1

https://pubchem.ncbi.nlm.nih.gov/compound/121892

Use of the compound

Retigabine is an anticonvulsant that binds to a Kv7 (KCNQ) channel and opens this voltage-gated potassium channel. It is used as an additional treatment to treat hyperexcitability, epilepsy. (Rundfeldt et al., 1997; Rogawski and Bazil, 2008) KCNQ isoforms, especially the subtypes KCNQ4 and KCNQ5, are located in smooth muscle cells. They are known to be engaged in forming and keeping the resting membrane potential and controlling the contractility of smooth muscle. (Wang et al., 2019)

The mechanism of action of Retigabine is different from other anticonvulsants but similar to the mechanism of flupirtine, which is chemically similar to Retigabine and has analgesic properties. (Brown and Passmore, 2009) In preclinical tests, Retigabine was found to be

effective in almost all animal models of epilepsy and seizures. A double-blind, randomised, and placebo-controlled Phase II clinical trial was conducted, where Retigabine was added to the basic therapy of the patients with partial seizures. The frequency of seizures significantly decreased with the administration of Retigabine. (Ben-Menachem, 2007)

A limiting factor of its application is the inadequate selectivity for the different subtypes of the channel. Wang et al. were able to generate derivatives of Retigabine, improving their subtype specificity by changing the N-1/3 substituents. (Wang et al., 2019)

Due to its low selectivity, Retigabine can cause severe side effects, such as urinary retention, retinal abnormalities and blue skin discolouration. (Jankovic and Ilickovic, 2013) Another reason for side effects are metabolites of its aniline ring. So, the FDA (U.S. Food and Drug Administration), restricted its use just to patients who do not get a response to other treatment options. (Kumar et al., 2016) For instance, Retigabine also activates KCNQ4, which is not implicated in hyperexcitability but is expressed in smooth muscle cells of the bladder, where it controls contractility. When KCNQ4 is opened, it causes membrane hyperpolarisation and reduces contractility, which is possibly the reason for urinary retention. (Greenwood and Ohya, 2009; Kumar et al., 2016)

Binding Site

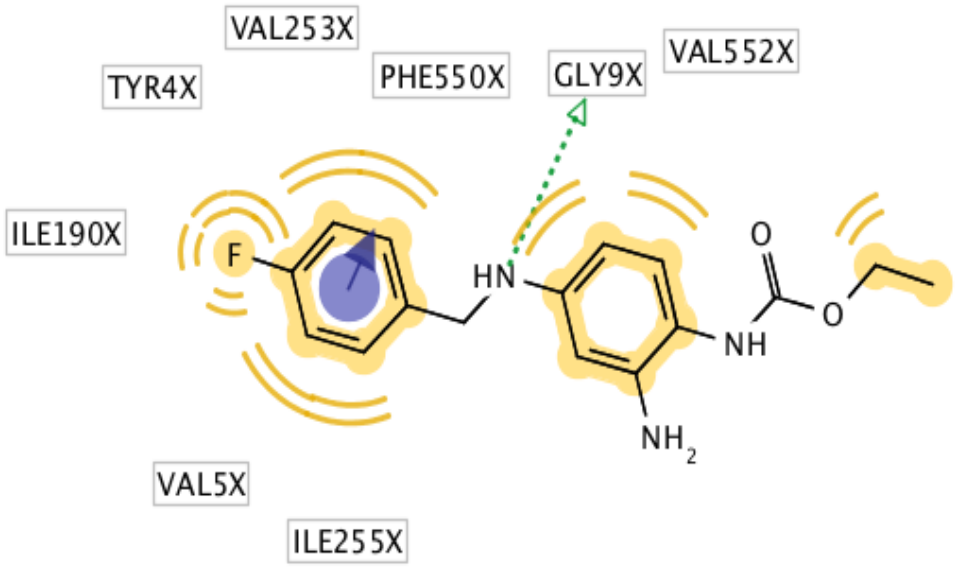


Figure 37: Pharmacophore model of RTG bound to GIRK2 (PDB: 4KFM)

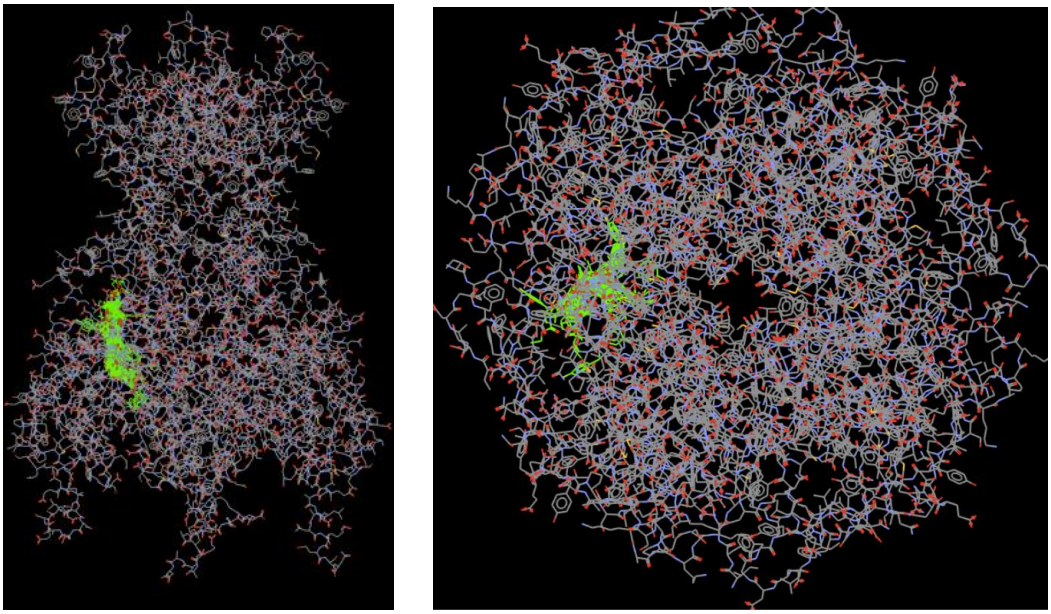


Figure 38: A side (left) and top-down (right) view of RTG bound to the cytosolic domain of GIRK2 (PDB: 4KFM).

The most favourable position for Retigabine is in the cytosolic part of the broader 4KFM structure. Its Estimated Affinity is between mM and low μM, but it does not block the pore directly, because it does not bind in the middle.

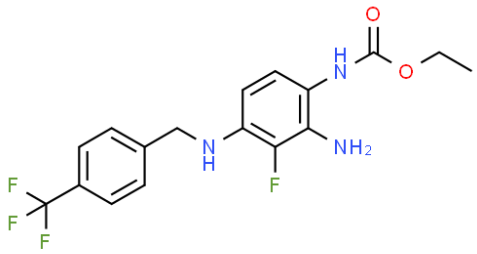
Retigabine has an aromatic ring forming pi-pi molecular interactions with Phe550, and it also has many hydrophobic properties that interact with hydrophobic amino acids, such as Val5, Val253, Val552, Ile190 and Ile255.

How to validate this hypothesis?

By classical mutagenesis, it can be experimentally tested, if Retigabine binds to the disease mutant. The aromatic amino acid phenylalanine can again be mutated to alanine. The other way is to mutate the hydrophobic amino acid valine into the polar amino acid threonine, to prevent hydrophobic interactions.

5.2.6. RL648-81

The compound



$C_{17}H_{17}F_4N_3O_2$

Ethyl (2-amino-3-fluoro-4-((4-(trifluoromethyl)benzyl)amino)phenyl)carbamate

Retigabine analogue

Molecular Weight	371.33 g/mol
Hydrogen Bond Donor Count	4
Hydrogen Bond Acceptor Count	5
Rotatable Bond Count	7
Topological Polar Surface Area	76 Å²
Covalently Bonded Unit Count	1

http://www.chemspider.com/Chemical-Structure.58825114.html

Use of the compound

KCNQ channels are voltage-gated K^+ channels, and their downregulation is known to be a cause in different diseases related to hyperexcitability, such as epilepsy, tinnitus and neuropathic pain. Compounds, which activate and open this channel reduce the excitability of nerve cells and can be used as drugs. RL648-81 is a derivative of retigabine, an anticonvulsant. A CF3-group was introduced at the 4-position of the benzylamine, and another fluorine atom was added at the 3-position of the aniline ring. Rl648-81 is a novel KCNQ2/3-specific channel opener, which is more selective and >15 times more effective than Retigabine, making it a promising drug candidate for the treatment of neurological disorders that are connected to neuronal hyperexcitability. The most frequent of all neuronal hyperexcitability disorders is epilepsy, a disease that affects around 1% of the population. (Kumar et al., 2016) Epilepsy is often treated with Na^+ channel blockers or GABA receptor

agonists. (Bialer et al., 2010) Even though there are more drugs and various mechanisms of action, a large number of patients (30%) do not benefit from these drugs, making it necessary to search for more treatment alternatives. (Sharma et al., 2015; Kumar et al., 2016)

Due to the improved specificity of RL648-81, it is believed to cause fewer side effects. It is also expected to be more resilient to photodegradation and consequently not as toxic to the skin and the eye. (Kumar et al., 2016)

Binding Site

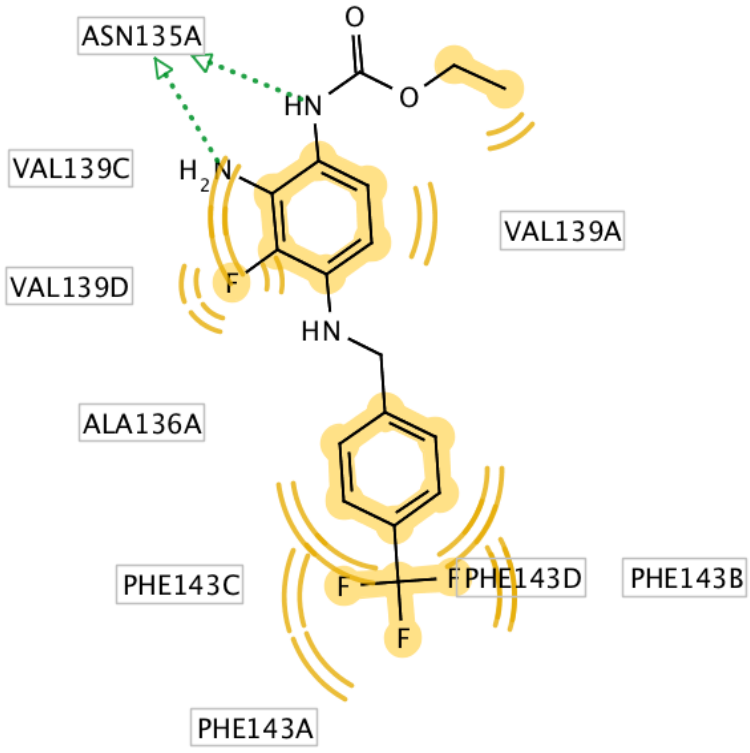


Figure 39: Pharmacophore model of RL648-81 bound to GIRK2 (PDB: 3SYA)

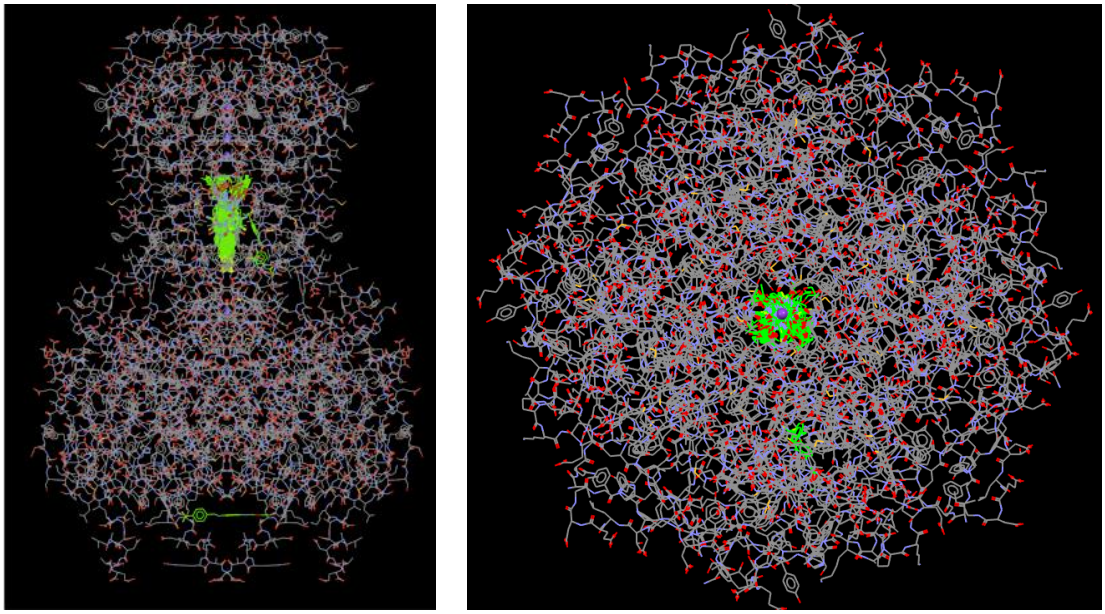


Figure 40: A side (left) and top-down (right) view of RL648-81 bound to the transmembrane region of GIRK2 (PDB: 3SYA).

The compound RL648-81 binds best to the central transmembrane cavity of the narrower 3SYA structure. It has an Estimated Affinity close to this of Retigabine, but slightly better.

This compound is mainly hydrophobic, due to both of its aromatic rings, the short alkyl chain and also because it is fluorinated. Several interactions are formed with the hydrophobic amino acids Ala136, Val139 and Phe143.

How to validate this hypothesis?

To examine if RL648-81 would bind to the disease mutant classical mutagenesis can be performed. Hydrophobic amino acids such as valine can be mutated to a polar amino acid like threonine, avoiding the formation of hydrophobic interactions.

6. Discussion

In 2020 De la Rosa and Shapiro published an article, where six compounds were proven to block GIRK2, an inwardly rectifying potassium channel, which maintains the resting membrane potential of a cell. These six compounds are XE991, M084, ML204, ML297, RTG and RL648-81.

In three patients with the Keppen-Lubinsky syndrome, mutations in the KCNJ6 (GIRK2) gene were identified. Another patient with a similar phenotype, but without lipodystrophy and epilepsy, suffered from a gain-of-function mutation in the same KCNJ6 gene.
This mutation alters the channel function, making it no longer G-protein-coupled and leading to a loss of K^+ ion selectivity, causing other cations to permeate through the channel. These changed channel properties produce sustained depolarisation; consequently, GIRK2 does no longer generate its normally inhibitory response.

Compounds, which can block the disease mutant are promising drug candidates, to treat patients suffering from this gain-of-function mutation in the KCNJ6 gene, because they may block these abnormal inward currents, responsible for the symptoms.

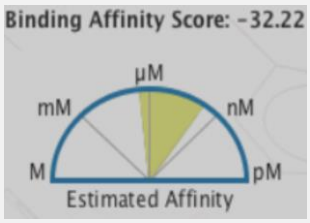
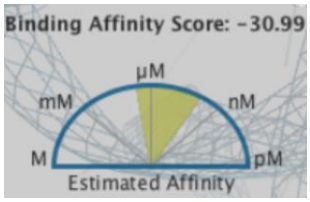
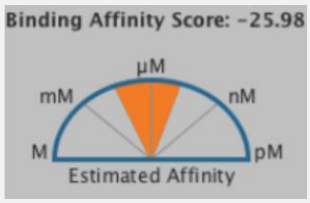
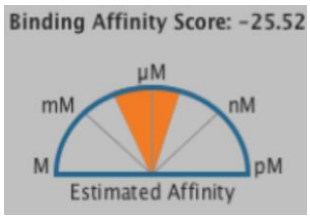
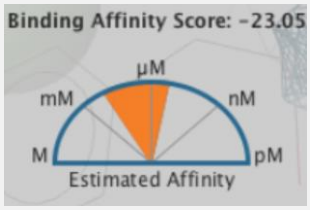
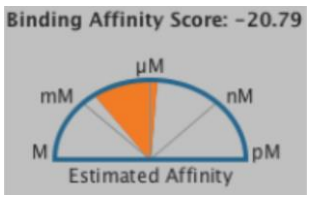
But the results are not just interesting for this p.Leu171Arg gain-of-function mutation. Other mutations in the KCNJ6 gene, causing the Keppen-Lubinsky syndrome may also benefit from channel inhibitors.

To determine the most probable binding site for each of these six compounds, they were docked in the transmembrane region and the cytosolic part of two crystal structures of GIRK2, using GOLD. These two crystal structures are 3SYA, a structure of GIRK2 in complex with Na^+ and PIP2, and 4KFM, a crystal structure of GIRK2 in complex with $G\beta\gamma$ subunits.

For all the poses with a high docking score, a pharmacophore model was created by the use of LigandScout. Based on their Estimated Affinity, the most favourable binding position was chosen and further analysed.

Ranking of the compounds

The following table ranks the six compounds from the highest to the lowest Estimated Affinity.

	ESTIMATED AFFINITY	BEST BINDING AFFINITY
XE991		Transmembrane region 4KFM
ML297		Transmembrane region 3SYA
RL648-81		Transmembrane region 3SYA
RTG		Cytosolic part 4KFM
ML204		Transmembrane region 4KFM
M084		Transmembrane region 3SYA

The compounds XE991 and ML297 show the highest affinity scores, both being in the μM range. ML297 seems to be a perfect inhibitor due to its high affinity and the way it blocks the pore exactly in the middle.

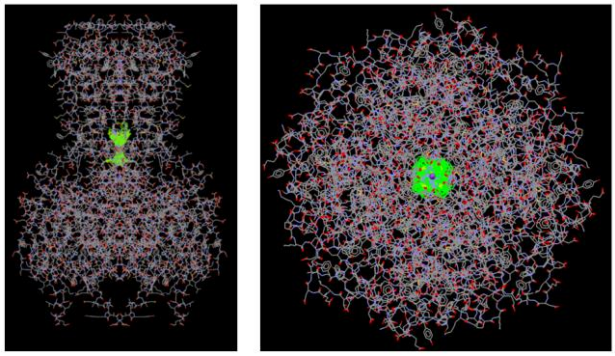


Figure 41: A side (left) and top-down (right) view of ML297 bound to the transmembrane region of GIRK2 (PDB: 3SYA).

RL648-81 follows, having an Estimated Affinity between mM and low μM, slightly higher than Retigabine. But Retigabine does not block the pore directly. It is the only one of the six compounds that binds in the cytosolic part of the channel and is not located in the middle of the pore.

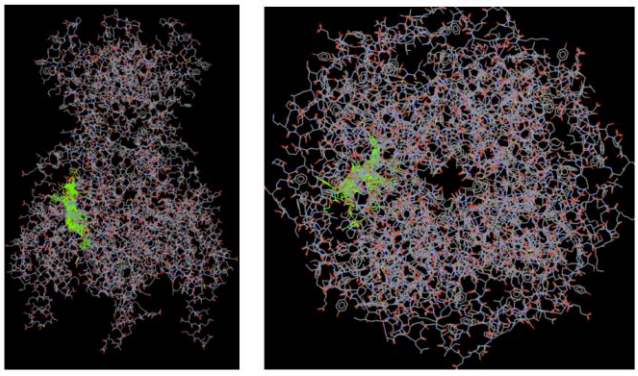


Figure 42: A side (left) and top-down (right) view of RTG bound to the cytosolic domain of GIRK2 (PDB: 4KFM).

ML204 and M084 showed the lowest Estimated Affinities out of the six compounds, both being in the mM range.

Experimental validation strategy

To examine if these compounds inhibit the disease mutant, experimental studies need to be conducted.

Classical mutagenesis can be performed, changing specific amino acids in GIRK2, that are essential for the compound binding.
Because all the compounds seem to form hydrophobic interactions, changing hydrophobic amino acids, such as valine or alanine, to the polar amino acid threonine, should prevent the formation of these interactions, which are essential for the compound binding.
Another way is to mutate the aromatic amino acid phenylalanine to alanine so that no pi-pi molecular interactions can develop.

An important aspect for future studies is to perform experimental validation, to find out if the compounds actually bind the disease mutants as well.
Experimental validation is necessary because these inhibitors seem to be interesting and promising drug candidates for the treatment of neurological disorders that are connected to neuronal hyperexcitability.

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